INTRATRACHEAL INOCULATION OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS IN MONKEYS *MACACA RHESUS*

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Received November 14, 2006; accepted August 17, 2007

Summary. – An animal model for infection with Severe acute respiratory syndrome coronavirus (SARS-CoV) was evaluated in monkeys *Macaca rhesus*. The monkeys were inoculated into the trachea with NS-I strain of SARS-CoV and the clinical manifestation of the illness was monitored. The clinical samples collected from infected monkeys were examined by immumnofluorescence assay (IFA), pathological inspection, RT-PCR, and by virus isolation. The infected animals demonstrated mild clinical symptoms including fever. Two of the six infected monkeys developed fever (1.5 above the level before challenge) on the day 10 post inoculation (p.i.). Although the severe clinical symptoms or mortality were not observed, the virological and histopathological evidences of the illness were evident. The specimens collected from the infected animals showed the presence of SARS-CoV detected by RT-PCR, IFA, and by virus isolation. From the organs examined postmortem, a major pathological change was observed in the lungs. The walls of the alveoli were thicker, infiltrated with inflammation cells and an exudative fluid was found in the alveolar spaces. In addition, some alveolar spaces showed hyaline membrane lining. The results showed that the monkeys infected with SARS-CoV developed the typical SARS according to clinical, virological, and pathological findings.

Key words: Severe acute respiratory syndrome coronavirus; animal model; *Macaca rhesus*

Introduction

The first emerging life-threatening and highly contagious epidemic of the 21st century was SARS spreading to more than 30 countries across five continents with excess of morbidity and approximately 10% mortality (Rota *et al*., 2003; Peiris *et al*., 2003; World Health Organization, 2003). Firstly, SARS was reported in the Guangdong province of China in November 2002 and the outbreak of this apparently new infectious disease came to the end in July 2003. The virus caused over 8600 cases worldwide with more than 700 deaths (Tai, 2006).

The mortality rate resulted at least in part from the absence of a definitive treatment protocols or therapeutic agents (Holmes, 2003). Although the virus spread was controlled, the preparedness and the successful development of antiviral drugs against SARS-CoV are necessary in case of possible reappearance of SARS. The understanding of SARS outbreak is very important for the drug development and for the managing of the new outbreaks that would emerge in the future (Heyman, 2003). Therefore, the available strategies for antiviral drug screening and use of these technologies for identification of potential antiviral agents against SARS-CoV are urgent. The classic approach using animal model may be the best method for evaluation of the new drugs and vaccines.

Most coronaviruses replicate in epithelial cells of the respiratory or enteric tract, where they produce virus and cause local respiratory symptoms or diarrhea that can be particularly severe in young animals. In humans, SARS-CoV infection always resulted in a serious lower respiratory tract

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Abbreviations: SARS = severe acute respiratory syndrome; SARS-CoV = SARS coronavirus; IFA = immunofluorescence assay; p.i. = post inoculation

illness. In this study, we reported the evaluation of the *M. rhesus* model for challenge with NS-I strain of SARS-CoV. We followed replication of the virus in various organs of infected monkeys and used a combination of clinical observation, histological examination, RT-PCR and direct isolation of the virus in cell culture for the virus detection.

Materials and Methods

Animals. The work with infectious virus and infected animals were performed in the biosafety level 3 facility (BSL-3). Animal protocols used in these studies had been approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases. Twelve 2–3-year-old monkeys *M. rhesus* (6 males and 6 females) were obtained from Yunnan Animal Cultivation Center in Yunnan with the weight about 5 kg. The animals were healthy and negative for SARS-CoV IgG. They were labeled No. 1–12, housed in single cage in BSL-3 at constant room temperature and arranged into 2 groups, signed as infected and non-infected, respectively. Before inoculation, the animals were anesthetized intramuscularly with ketamine hydrochloride (5 mg/kg of body weight). Then, monkeys in the infected group were inoculated with a dose of 0.5×10^8 PFU/animal in volume of 0.5 ml by direct instillation into the trachea. The non-infected monkeys were inoculated with PBS instead of virus.

Virus and cells. The SARS-CoV, strain NS-I is the Chinese representative virus (No. SH200301334, Detection station of medicine and biological products) isolated from the urine of SARS-patient in province Ningxia of China. The titer of the virus stock was 1×10^8 PFU/ml. Vero cells (Wuhan University) were grown in Minimum Essential Medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 mg/ml), sodium bicarbonate (0.2%), and L-glutamine (2 mmol/l).

Collection of samples. The samples were collected on different day p.i. The venous blood was collected on days 2 and 5 p.i. and obtained serum was stored at -20°C. Nasopharyngeal and rectal secretions were collected with aseptic swabs on day 3, 5, 7, and 9, p.i., washed in 1 ml PBS, and centrifuged at 400 × g for 5 mins. The supernatants were stored at -20°C. The urine samples were collected every other day p.i. The feces were collected on day 3, 5, 7, and 9, p.i. Necropsy was conducted on days 10 and 14 p.i. and tissue samples were removed from the lungs, kidneys, liver, heart, and brain for pathological examination. The tissues were fixed in formalin, embedded, sectioned and stained with hematoxylin and eosin and the histological sections were observed in a blinded fashion for histopathology using a light microscope. Additionally, residual lung samples were frozen and used for further examination. To minimize cross-contamination between samples during the necropsy, we used the disposable tools for removing of the tissues and sample processing.

Clinical observation. After inoculation, the animals were inspected daily for the presence of clinical symptoms, such as change in activity, rectal temperature, rash, etc. Before sacrifice, the chest X-ray examination was performed for all animals at the posterior-anterior position.

Virus isolation. Filtered nasopharyngeal and rectal washings, serum, or 10% tissue homogenates were added in volume of 100 µl in 24-well plates with monolayer of Vero cells (Costar). After incubation for 6–8 hrs, the inoculum was replaced with complete MEM containing 2% FBS. The cells were observed for 7 days and passaged three times consecutively until the viral CPE was observed. The specimens giving no evident CPE were assayed for the presence of viral antigen by immunofluorescence assay.

Immunofluoresence assay (IFA). The smears of infected Vero cells were fixed by cold acetone and incubated with 100 µl of inactivated convalescent serum diluted 1:100 with PBS obtained from a patient with atypical pneumonia for 30 mins at 37°C. Then, the cells were washed with PBS and incubated for 20 mins at room temperature with 100 µl of FITC-conjugated goat anti-human IgG. Finally, the cells were washed, dried, mounted, and examined under fluorescence microscope. The cell structures with bright green fluorescence were considered as positive and the reddish-yellow ones as negative.

Detection of viral RNA. The presence of the virus in the blood, nasopharyngeal swab, feces, urine, and lung homogenate was detected by RT-PCR. Total RNA kit (Promega) and Trizol kit (Invitrogen) were used for RNA extraction. The random primers were used for the preparation of complementary DNA templates for PCR. The forward and reverse primers used for RT-PCR were 5'-CA GAGTTGTGGTTTCAAGTG-3' (nt 21431 to 21450) and 5'-CA CAGAGTAATCAGCAACAC-3' (nt 22538 to 22519), respectively. The reverse transcription step was conducted at 43°C for 1 hr and at 95°C for 5 mins. Amplification of the cDNAs was performed at 94°C for 2 mins, followed by 35 cycles of 94°C for 10 secs, 55°C for 30 secs, and 68°C for 3 mins, after that extending at 68°C for 7 mins. The tissue samples from the non-infected monkeys were used as the negative control and the SARS-CoV NS-I strain was used as the positive control. The amplicon (1.1 kb) for SARS-CoV was resolved in 1% agarose gel and visualized using ethidium bromide (0.5 µg/ml) under UV light (Wu *et al*., 2004).

Results

Clinical signs

After virus inoculation, no severe illness symptoms as coughing, skin rashes and respiratory distress were observed in infected animals. Although no evident clinical respiratory symptoms appeared, minor lethargy and loose stools were observed on day 3 to 5 p.i. Monkeys No. 3, 4, 5, and 6 showed mild change in body temperature. The monkeys No. 1 and 2 developed fever (1.5°C above the level before challenge) on day 10 p.i. The body weight of the monkeys decreased slightly accompanied with the increased fever. Non-infected monkeys had no clinical symptoms. None of the infected or non-infected monkeys died till the day 10 or 14 p.i.

Radiographs

The chest radiographs were taken for each infected and non-infected animal. The monkey No. 2 exhibited the radiographic evidence of pulmonary disease and developed a left upper lobe infiltrate accompanied with a slight increase of opacity in the right lower lobe (Fig. 1B). Chest radiograph taken from a non-infected monkey was normal (Fig. 1A).

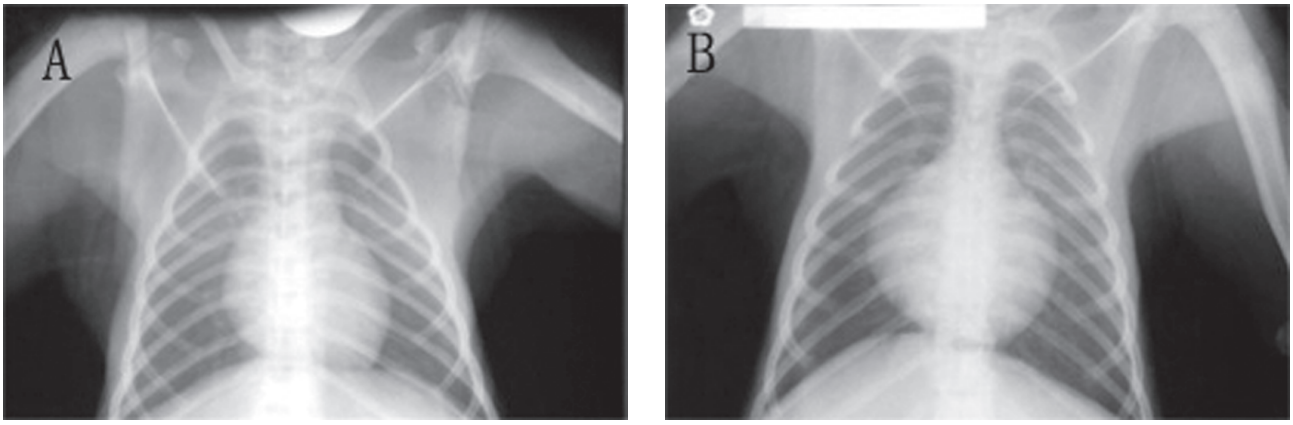

Fig. 1

Chest radiograph of *M. rhesus* non-infected (A) and infected with SARS-CoV (B)

Detection of SARS-CoV in infected Vero cells by virus isolation or by IFA

The isolation of infectious virus was performed from various samples collected from infected monkeys at different time p.i. The samples of lungs, blood, nasopharyngeal and rectal swabs taken from infected animals were inoculated to monolayers of Vero cells and produced typical CPE. The virus was detected in lung tissues removed from 4 infected monkeys (No. 1, 2, 3, and 5). CPE was first observed in the cells infected with nose-throat secret taken between day 3–5 p.i. from monkeys No. 1 and 2 after the first passage. The specimens showing no evident CPE after triple blind passage were tested for the presence of viral antigen in IFA (Table 1). The viral antigen was detected in the cells infected with the lung tissue homogenates collected from all infected monkeys showing positive fluorescence in cell membrane (Fig. 2).

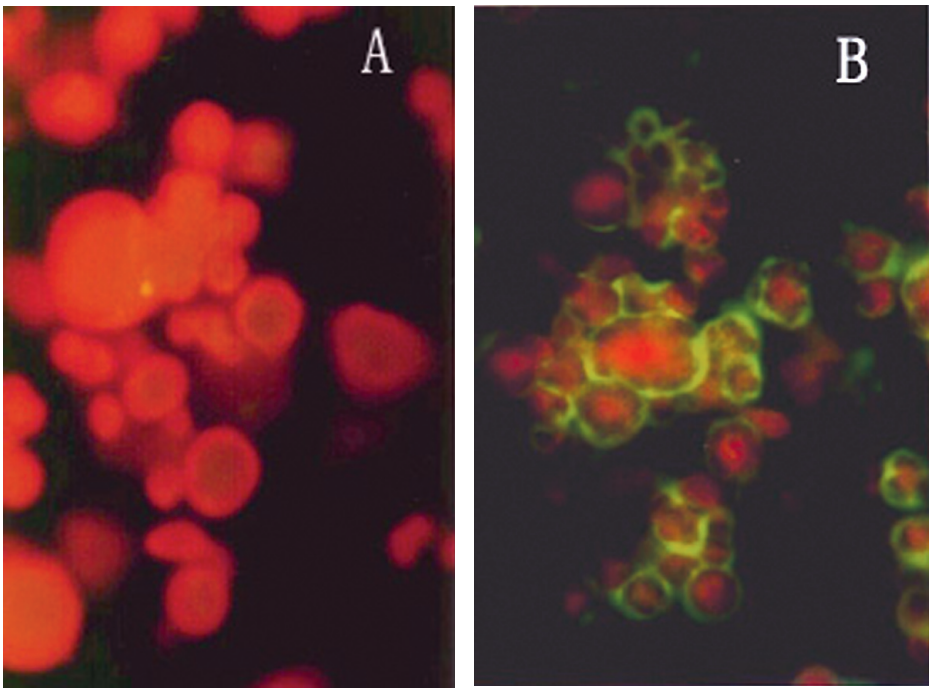

Fig. 2

IFA of Vero cells non-infected (A) and infected with lung homogenates of SARS-CoV infected monkeys (B)

Magnification 400x (A) and 200x (B).

Table 1. IFA detection of SARS-CoV in various tissues of infected and non-infected monkeys *M. rhesus* on different day p.i.

Group	Gender	No.	Day p.i.																
			Nose-throat secret				Feces				Urine					Blood		Lungs	
			3	5	7	9	3	5	7	9	1	3	5	7	9	2	5	10	14
Infected	M	1	–	±	++	+	–	–	–	–	–	–	–	–	–	–	+	+++	ND
	F	2	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+	++	ND
	F	3	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	ND
	M	4	++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	±
	F	5	-	+	+	–	–	–	–	–	–	–	–	–	–	–	–	ND	+++
	M	6	++	+	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	+
	F	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
	F	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
	F	9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
Non-infected	M	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
	M	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
	M	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–

Intensity of fluorescence: (+++) = bright green, (++) = yellow green, (+) = weaker, (±) = extremely weak, (–) = none, ND = not detected.

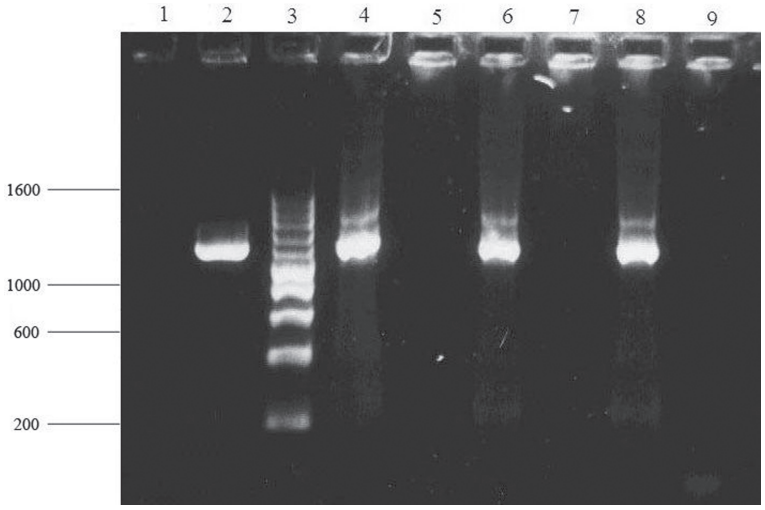

Fig. 3

RT-PCR analysis of RNA of SARS-CoV

Blank (1), strain NS-I (2), DNA size marker, 200 bp ladder (3), the mixture of feces and urine, nose-throat secret, and blood of infected (4, 6, 8, respectively) and non-infected monkeys (5, 7, 9, respectively).

Table 2. RT-PCR detection of SARS-CoV RNA in various tissues of infected and non-infected monkeys *M. rhesus* on different day p.i.

Group	Gender	No.	Day p.i.															
			Nose-throat secret				Feces				Urine					Blood		Lungs
			3	5	7	9	3	5	7	9	1	3	5	7	9	2	5	10
Infected	M	1	–	–	+	+	–	–	–	–	–	–	–	–	–	+	+	+
	F	2	–	–	+	–	–	+	–	–	–	–	+	–	–	+	+	+
	F	3	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+
	M	4	+	–	+	-	+	–	–	–	–	–	–	–	–	–	–	–
	F	5	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
	M	6	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
	F	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	F	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	F	9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-infected	M	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	M	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(–) = negative amplification; (+) = positive amplification.

Detection of SARS-CoV RNA by RT-PCR

The presence of specific amplification product (1.1 kb) was tested by RT-PCR in following samples: blood, secrets of nose-throat, mixture of feces and urines of infected and non-infected monkeys (Fig. 3). Nasopharyngeal secrets from the infected animals taken on day 3, 5, 7, and 9, p.i. were positive for the presence of SARS-CoV RNA. In addition, the viral RNA was found in the feces on day 3 and 5 p.i. and in the urine on day 5 p.i. in the infected monkeys. The blood of the infected monkeys on day 2 and 5 p.i. was also positive for the presence of SARS-CoV RNA. The lung of the infected monkeys on day 10 p.i. was positive. The rest of the fecal and urine samples were negative (Table 2).

Histopathological studies

The lungs from the infected monkeys displayed a consolidation in both sides due to the inflammatory induration and the presence of cellular exudates in alveoli. Diffuse alveolar damage was common in the infected animals. The alveolar wall was thickened and infiltrated with mononuclear cells and lymphocytes. Light-red exudate containing fibrin, erythrocytes, macrophages, and scattered epithelial cells was present in the alveolar spaces. In addition, some alveolar spaces showed hyaline membrane lining (Fig. 4). We did not observe any of the typical multinucleate syncytial cells and fibroblasts in the histological sections. The liver of the monkey No. 1 and 2 showed a local necrosis and infiltration with monocytes (Fig. 5). No significant pathological changes were observed in the other organs.

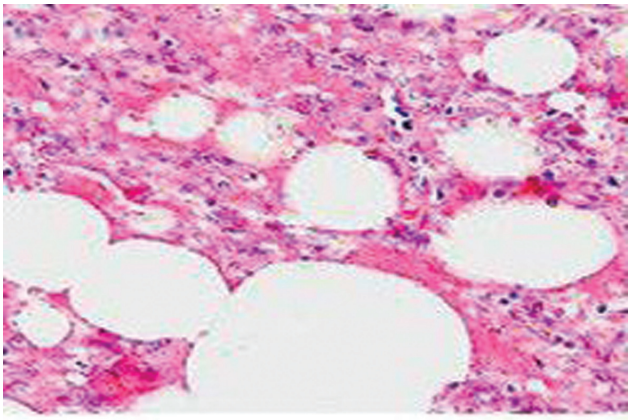

Fig. 4

Lungs of *M. rhesus* infected with SARS-CoV stained with hematoxylin-eosin

Magnification 400x.

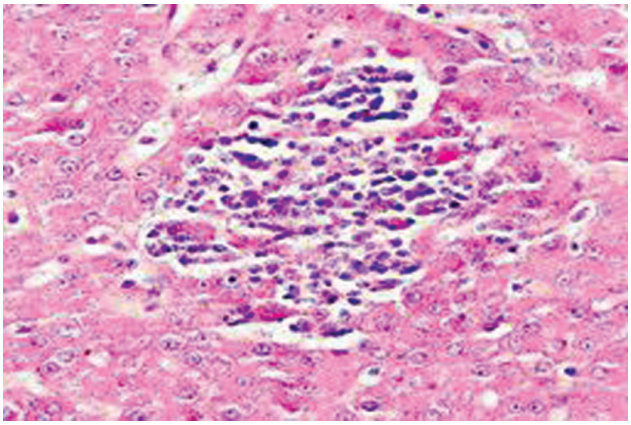

Fig. 5

Liver of *M. rhesus* infected with SARS-CoV stained with hematoxylin-eosin

Magnification 400x.

Discussion

Although in the year 2004, there were no significant SARS outbreaks and none of the few isolated cases resulted in death or secondary transmission, it was possible that resurrection of SARS would lead to a major epidemic. Therefore, the development of an effective vaccine or a drug against SARS-CoV is crucial in preventing future epidemics, what requires further refinement of animal model for elucidation of the pathogenesis of SARS.

An array of animal species is susceptible to the experimental infection with SARS-CoV including rodents (mice and hamsters), carnivores (ferrets and cats), and nonhuman primates (cynomolgus and rhesus macaques, common marmosets, and African green monkeys) (Kuiken *et al*., 2003; Roberts *et al*., 2005; Martina *et al*., 2003; McAuliffe *et al*., 2004; Rowe *et al*., 2004; Greenough *et al*., 2005; Bukreyev *et al*., 2004). Early studies using rodents were not successful in reproducing SARS clinical symptoms and/or pathology, but non-human primates showed great value as a model for SARS infection and pathology (Fouchier *et al*., 2003). The suitability of a monkey model for testing of the vaccine against SARS-CoV was described (Gao *et al*., 2003). According to the summary of "SARS ANIMAL MODELS", (Kuiken *et al*., 2003; Martina *et al*., 2003), which was held on February 5–6th 2004, SARS models of mice, ferrets and monkeys were available. However, the suitable animal model for following the clinical symptoms of the disease had to be developed. Some factors may affect the outcome of SARS-CoV infection of different animal models in different laboratories, such as time of the necropsy,

strain of the virus used, genetic background, specific pathogen free status, age of the animals, SARS-CoV challenge dose, and stress of the animals.

In this work, we inoculated six monkeys *M. rhesus* by the intratracheal route, which was different from the previously reported experiments using animals inoculated intranasally or intravenously (Roberts *et al*., 2005; Martina *et al*., 2003; McAuliffe *et al*., 2004; Rowe *et al*., 2004). Our studies included a 2–14 days life phase p.i. with an evaluation of clinical and virological analyses, followed by a thorough evaluation of the tissues removed at necropsy. Nasopharyngeal and rectal swabs, blood, feces and urine from the animals were collected at different day p.i. Finally, the monkeys from the infected and the non-infected group were sacrificed and their organs removed for pathological examination on different day p.i. In this experiment, no significant clinical symptoms were seen in the infected monkeys, except for two monkeys (No. 1 and 2) that showed transient fever. None of the six infected monkeys died after the challenge and demonstrated severe symptoms of respiratory illness or rashes. Similar clinical symptoms were observed with the infected African green monkeys, cynomolgus and rhesus macaques. These animals showed no marked clinical signs of disease, especially African green monkeys (McAuliffe *et al*., 2004). Likewise, clinical symptoms present in the adult mice infected with varying dose of SARS-CoV *via* respiratory tract, we found that three infected monkeys (No. 4, 5, and 6) released the virus in the nasopharyngeal secrets starting on day 3 p.i. Moreover, monkey No. 4 released the virus in feces on day 3 p.i. In our study, two monkeys (No. 1 and 2) showed viremia on day 2 p.i. and the virus could be isolated from the lungs tissue of four monkeys (No. 1, 2, 3, and 5). SARS-CoV RNA was detected by RT-PCR in different specimens obtained from monkey No. 1, 2, 3, and 5. The result of RT-PCR matched to the one of the following three items:

1) Specimens obtained from two different parts of the same organ were positive for viral RNA,
2) The same kind of specimen collected every other day proved as viral RNA positive,
3) Specimen should be found positive for viral antigen by two different methods or to be positive for viral RNA.

The results of our experiment matched to the above standards. Apparently, the SARS-CoV could replicate in monkeys *M. rhesus*.

Among organs removed at autopsy, the most important lesions occurred in the lung. The pathological characteristics were as follows: acute pulmonary exudation, hemorrhagic inflammation and type II pneumocytic hyperplasia, greatly increased permeability of capillaries due to the strong stimulation of pathogen. We observed the typical formation of hyaline membrane in the alveolus cavity, fibrous deposition in the alveolar walls, and as well as the presence of many inflammatory cells in the alveolus cavity. The walls of the alveoli were thickened due to the edema and type II pneumocyte proliferation. These diffuse alveolar lesions were quite similar to those occurring in SARS patients. The pathological changes showed that SARS-CoV might replicate in the lung and liver tissues indicating that SARS-CoV was a pathogen with higher virulence for lung and liver tissues. The SARS-CoV might bind to the virus receptors in the lung and liver tissues leading to wide exudation and hemorrhagic inflammation that could result in obstructed ventilation, anoxia, dyspnea, respiratory failure, and animal death. However, this study had some potential limitations, as it was not designed to achieve statistical significance in all parameters measured.

Our results demonstrated that monkeys *M. rhesus* were susceptible to experimental infection with SARS-CoV NS-I. According to the histopathological examination of postmortem tissue and viral assay, SARS-CoV NS-I inoculation of monkeys could produce similar pathology as SARS in humans and provided a useful animal model for SARS research. Furthermore, this model was already used for a pre-clinical evaluation of an inactivated vaccine candidate against SARS-CoV for immunogenicity, safety, and protectivity (Zhou *et al*., 2005).

Acknowledgement. This work was supported by the Medicine Foundation of China.

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