

Pathology of Guinea Pigs Experimentally Infected with a Novel Reovirus and Coronavirus Isolated from SARS Patients

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ABSTRACT

Guinea pigs were inoculated with a reovirus (ReoV) and coronavirus (SARS-CoV) isolated from SARS patients to determine their potential role in the etiology of SARS. Animals infected with ReoV died between day 22 and day 30 postinoculation (PI) while 70% of the animals inoculated with ReoV and SARS-CoV died between day 4 to day 7 PI. The titer of neutralizing antibodies against ReoV and SARS-CoV ranged from 80 to 160 when the animals were inoculated with the two viruses, respectively, while the titer of the antibodies was just below 10 in coinfections. The animal inoculated with ReoV developed diffuse alveolar damage similar to the exudative and leakage inflammation found in SARS patients, and was characterized by diffuse hemorrhage, fibroid exudation, hyaline membrane formation, and type II pneumocytes hyperplasia in alveolar interstitia. The pulmonary epithelial necrosis, excoriation, and early fibrosis of pulmonary tissue were only observed in ReoV–SARS-CoV groups and in SARS-CoV/ReoV groups. Other typical pathological changes included hemorrhagic necrosis in lymph nodes and spleen and hydropic degeneration in the liver. On the contrary, guinea pigs infected with SARS-CoV only developed interstitial pneumonitis. Our experiment demonstrate that ReoV might be one of the primary causes of SARS, since simultaneous coinfection can duplicate the typical pathological changes similar to that of SARS patients. This guinea pig model may provide a useful animal model for SARS.

INTRODUCTION

THE FIRST CASE of SARS was recognized in February 2003, in Hong Kong. Clinically, SARS is characterized by fever, dyspnea, lymphopenia, along with rapidly progressing changes on radiography (Yang *et al.*, 2004). A new coronavirus (SARS-CoV) has been consistently associated with this disease (Chan-Yeung and Yu, 2003; Lee *et al.*, 2003; Peiris *et al.*, 2003). Although the World Health Organization (WHO) declared that the SARS-CoV was the primary pathogen, the contagious pathogen could not be confirmed in 60% of the clinical cases in Beijing during the SARS outbreak in 2003 (Walgate, 2003). In our present study, four strains of ReoV were isolated from a SARS patient in March 2003, and confirmed to be the same virus by biological characteristics and antigenicity assays (Duan *et al.*, 2003; Zuo *et al.*, 2003). The purpose of this study was to determine the potential relationship between Reo and SARS. To

identify the role of the newly isolated ReoV, guinea pigs were inoculated with the ReoV and SARS-CoV according to different regimens, and the pathological characteristics were subsequently observed and evaluated microscopically.

MATERIALS AND METHODS

ReoV and SARS-CoV

Hep-2 cells were infected with 50 μ l of the ReoV strain (BYD1) ($10^{7.5}$ TCID₅₀ per 0.05 ml), which was isolated from a SARS patient in Beijing by Dr. Duan (Academy of Military Medical Sciences, Beijing). The SARS-CoV strain (BJF), provided by Dr. Zhu (Virus Research Laboratory, Academy of Military Medical Sciences), was replicated on Vero cells with an end titer of $10^{4.7}$ TCID₅₀ per 0.05 ml (Zhu *et al.*, 2003). The

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TABLE 1. SUMMARY OF GROSS LESIONS AND PATHOLOGY

Group	Lung consolidation	Histopathology in lung tissue			
		Diffuse alveolar damage	Hyaline membrane formation	Interstitial pneumoniti	Other pathological findings
1	++++	+++++	+++	RBC, fibrin leakage	diffuse hemorrhage
2	+	++	-	Type II pneumocytes hyperplasia, fibrin leakage	minor hemorrhage
3	++++	++++	++++	RBC, type II pneumocytes hyperplasia, fibroblast proliferation	“Hang lamp” structure in cavity
4	+	++	+	RBC, fibrin leakage	severe hemorrhage
5	+++	+++	++	type II pneumocytes hyperplasia, fibroblast proliferation	fibronus exudation

++++ and +++++: represents severe pathological change.

++: represents moderate pathological change.

+: represents average/mild pathological change.

-: no change.

“Hang lamp” means that proliferated fibroblast projected toward alveolus cavity and resulted in narrowness of the airway.

cell cultures were performed under biosafety level 3 conditions. All experimental protocols received prior approval from the Animal Care Commission of Beijing Laboratory Animal Administration, and the procedures were performed in accordance with the requirements of the guide on the care and use of experimental animal (China State Department on Laboratory Animal Regulation, 1988).

Animals

Ninety-six 70-day-old British strain guinea pigs (Laboratory Animal Institute of Academy of Chinese Medical Sciences, Beijing) were randomly divided into six groups. They were maintained on a standard commercial diet with food and water available *ad libitum* and then placed in negatively pressurized and individually ventilated cages.

Experimental treatments

The six groups were then subjected to different infection regimens. Group 1: infected by ReoV in at a range of serial dilutions

(10^0 to 10^{-3}). Group 2: inoculated with different dilution of SARS-CoV just as Group 1; Group 3: a simultaneous inoculation with 10^{-3} diluted ReoV and serial dilutions of SARS-CoV. Group 4: inoculated with 10^{-3} diluted ReoV initially, followed by a second inoculation with serial dilutions of SARS-CoV. Group 5: initial inoculation with different dilutions of SARS-CoV and subsequently inoculated with 10^{-3} diluted ReoV. Group 6: inoculated with sterile physiological saline as a control group. All inoculations were by an intraperitoneal injection (i.p.) of 1 ml per animal. Clinical symptoms and mortality rates were then ascertained.

Antibody assays

On day 37, sera were collected and each was divided into two samples. Antibodies against ReoV and SARS-CoV were evaluated by microneutralization assays. One of the samples was mixed with 100TCID₅₀ of ReoV; another was blended with 100TCID₅₀ of SARS-CoV (BJF), and incubated for 1 h at 25°C.

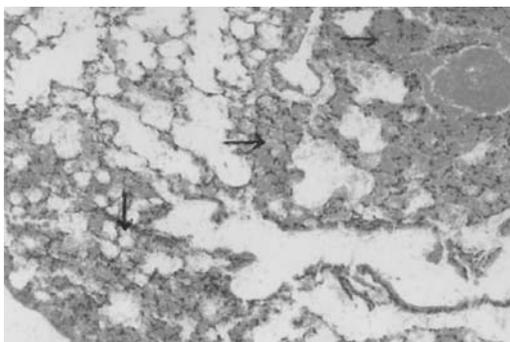


FIG. 1. Lung section from a guinea pig 33 days after ip injection with a 10^{-3} dilution of ReoV showing wide alveolar damage and hemorrhage in the alveolar cavity (arrow).

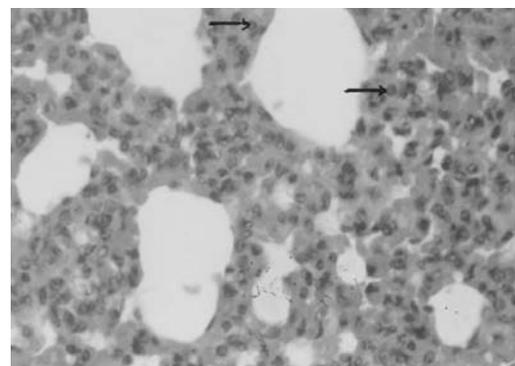


FIG. 2. Lung section from a guinea pig 33 days after ip injection with SARS-CoV, showing that the alveolar walls were moderately thickened and lined by type II pneumocyte hyperplasia (left arrow).

Subsequently, 50 μ l of each of the mixtures was added to a 96-well tissue culture plate in triplicate. Hep-2 cells or Vero-E6 cells were added to each antibody/virus mixture, and the plate was incubated at 37°C in the presence of 5% CO₂ for 3–4 days. The plates were then sealed and incubated at 37°C until the full cytopathic effect (CPE) was observed in the virus controls. The end point of the microneutralization assay was defined as the dilution at which >50% of the testing wells are not protected from infection; in other words, the end point titer is reached when three, or two of the three wells are not protected. ReoV and SARS-CoV alone served as positive controls for the neutralization.

IgG antibodies against SARS-CoV and ReoV were detected by ELISA kits (Huada Bioscience, China). The positive results was evaluated according the standards: OD values of the sample is more than 0.2, and OD values of the sample/negative sample is >2.0.

Histopathology and virus isolation

Necropsies were performed on the surviving guinea pigs according to a standard protocol. The guinea pigs were euthanized and subjected to gross examination. Samples of lung, liver, kidneys, and spleen were collected and fixed in a 10% formaldehyde solution before being embedded in paraffin; 5 μ m tissue sections were prepared and routinely stained with hematoxylin and eosin. The lung tissues for virus isolation were frozen and then thawed three times in 2 ml of 0.5% gelatin dissolved in phosphate-buffered saline, followed by homogenization with tissue grinder, and sonication with a microprobe tip. After centrifugation, serial dilution of tissue homogenates were used to inoculate onto Hep-2 cells cultured at 33°C with 1640 culture medium. The second generation of virus was obtained when obvious CPE was observed and then defined by RT-PCR.

RESULTS

Clinical signs

In Group 1, the guinea pigs were in poor spirit, had lower appetites, diarrhea, and bled from the nose, and total death oc-

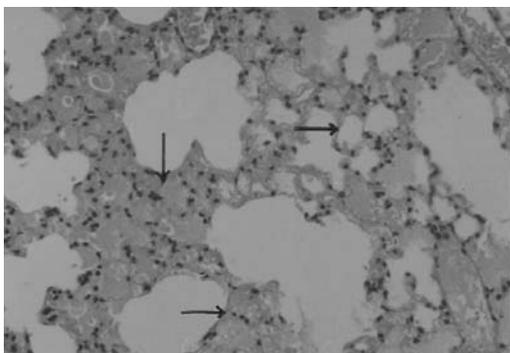


FIG. 3. Lung section from a guinea pig 33 days after ip injection with a 10⁻³ dilution of ReoV and 10⁻¹ of SARS-CoV showing marked hyaline-membrane formations in the alveolar lumina (arrow).

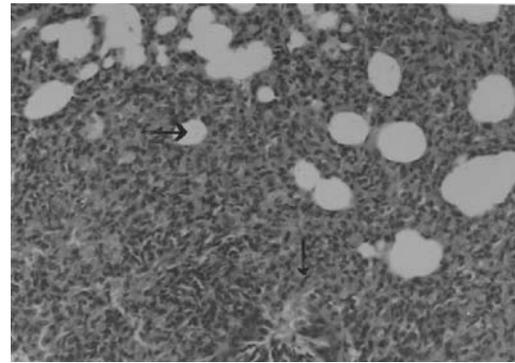


FIG. 4. Lung section from a guinea pig 33 days after ip injection with a 10⁻³ dilution of ReoV and 10⁻² of SARS-CoV showing fibroblast proliferation projected toward the alveolar lumina (arrowhead) with resultant narrowing of the alveolar cavity (arrow).

curred after postinoculation (PI); particularly, the animals inoculated with the primary titer died between days 22 and 24 PI. The guinea pigs died between days 22 and 28 inoculated with the 10⁻¹ dilution and the 10⁻² dilution of ReoV. The animals inoculated with the 10⁻³ dilution of ReoV died on days 25 to 30. In Group 3, those that were infected with SARS-CoV and 10⁻³ diluted ReoV all became dead between days 3 and 7 after PI. The earliest death occurred on day 3. The mortality rate was 3 of 4, 2 of 4, and 1 of 4 in the following combinations such as 10⁻³ ReoV+ SARS-CoV, 10⁻³ ReoV+10⁻¹ SARS-CoV, and 10⁻³ ReoV+ 10⁻³ SARS-CoV, respectively. Of course, the survivors increased while the SARS-coV titer decreased (Table 1). No apparent clinical signs and no mortality cases occurred in Groups 2, 4, and 5.

Serum antibody

Neutralizing antibodies against ReoV and SARS-CoV in Groups 1 and 2 had titers from 80 to 160, while the antibody was below 10 in Groups 3, 4, and 5. In ELISA assays, the antibody titer against ReoV was between 80 and 1280 in Groups 1, 3, 4, and 5, with the antibody against SARS-CoV from 320 to 2048 in Groups 2, 3, 4, and 5. The antibody titer gradually elevated as we lowered the inoculation dilution of SARS-CoV.

Gross lesion

The guinea pigs in Groups 1 and 3 had multiple foci of pulmonary consolidation in both lungs. The infected lung tissue was purple-red, firm, and less buoyant than normal. Hemorrhage was also widely evident in Group 5. Other lesions were as follows: some white necroses on the surface of the livers, and an enlarged size of the tracheobronchial lymph nodes and spleen. In addition, atypical lesions could be observed in other groups.

Histopathological changes in the lung tissue

Some sections of lungs in different areas showed exudation (Table 1). In Group 1, the characteristic histopathology was the

acute exudation, and leakage resulting in inflammation, such as the light-red exudative fluid containing fibrin, erythrocytes, and epithelial cells, was distributed in many alveolar interstitial. Typical hyaline membranes formation in the alveolus cavity, and fibrous deposition in the alveolar walls could be observed (Fig. 1), but scanty inflammatory cells could be seen in the alveolus cavity. The walls of the alveoli were thickened due to edema and type II pneumocyte proliferation. The capillaries of the alveolar wall were highly dilated and congested with associated edema with some fibrous thrombosis. In Group 2, the thickness of the alveoli walls was due to the type II pneumocytic hyperplasia and the interstitial infiltrates of fibrin (Fig. 2). The pulmonary alveoli showed a pattern of less acute lung injury characterized by less hemorrhage, hyaline membranes, and scattered proliferated fibroblast. Not only oxidative, proliferative inflammation, but also fibrous inflammation could be found in Groups 3 and 5. The typical hemorrhage, exudation, and hyaline membranes formation in alveolus cavity was obviously seen (Fig. 3), particularly in the groups inoculated with ReoV (10^{-3} dilution) and SARS-CoV (10^{-1} dilution). With the lower titer of SARS-CoV, the formation of hyaline membranes disappeared, and interstitial pneumonitis became more apparent instead. The proliferation of type II pneumocytes and fibroblasts in alveolus resulted in fibrosis in the lung. Fibroblast notably proliferated in the alveolus diaphragm, projected toward the alveolar cavity, and resulted in narrowness of the alveolus cavity, just like a "hang lamp" (Fig. 4). About 2/3 of the alveolus walls evidently became thick in Group 5. Massive fibrous exudation, scattered type II pneumocytes, and a few fibroblasts were noted in intraalveolar tissue. The mucosa of the bronchioles partly fell off, and the lymphocytes infiltrated in the lamina propria. There were many infiltrations of lymphocytes around the bronchi. On the contrary, hyaline-membrane formation was rare in the alveolus cavity in Groups 4 and 5.

Histopathological changes in other extrapulmonary organs

Furthermore, in all treated groups, the normal structure of lymph nodes at the lung hilum disappeared with severe con-

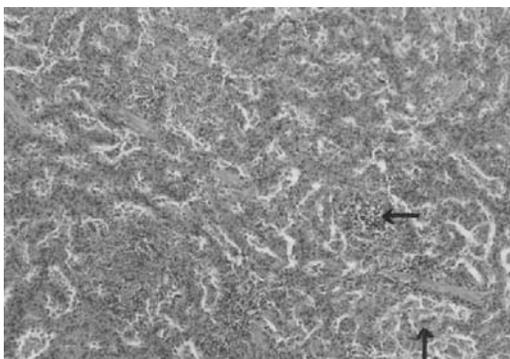


FIG. 5. Spleen section from a guinea pig 33 days after ip injection with a 10^{-3} dilution of ReoV and 10^{-2} of SARS-CoV showing reduction in lymphocytes around the lymphatic sheath (left arrow) and hemorrhagic necrosis in the capsule (up arrow).

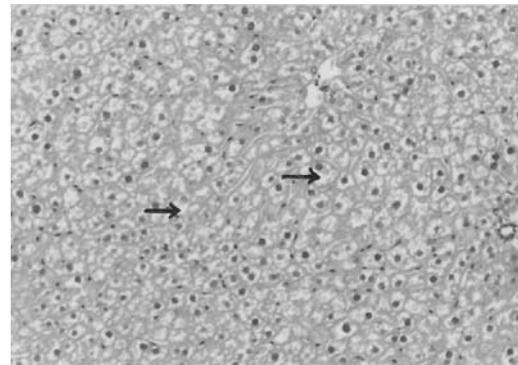


FIG. 6. Liver section from a guinea pig 33 days after ip injection with a 10^{-3} dilution of ReoV and 10^{-2} of SARS-CoV showing light hepatocytic swelling and fatty degeneration in the lobules of the liver (arrow left). All slides were stained with hematoxylin and eosin.

gestion in the central artery, trabecular vein, and trabecular artery. The lymphocytes in cortices were greatly reduced, and the germinal center disappeared. The changes in spleen, apparent by microscopy, included the atrophy of white pulp, the reduction of lymphocytes around the lymphatic sheath, and the rarefication of lymphocytes in red pulp (Fig. 5). The focal hemorrhagic necrotic inflammation could be observed near the capsule. The hepatocytes showed light swelling and fatty degeneration in lobules of the liver. The Kupfer's cells proliferated actively and the portals expanded slightly with a few lymphocytes infiltrated. The other hepatocytic lesions included vacuolar and hydropic degeneration in the cytoplasm (Fig. 6). Interlobular veins and central veins were congested, and the light-red and fibrous deposition could be observed. The small intestine and stomach had no evident pathological changes. No obvious pathological lesion was found in the kidney, except for the congested blood in the intertubular veins.

DISCUSSION

In our experiments, animals infected with ReoV solely as well as ReoV and SARS-CoV concurrently induced mortality. Meanwhile, the coinfection of the ReoV and SARS-CoV can accelerate the mortality only on day 3.

Guinea pigs infected with ReoV alone or with both ReoV and SARS-CoV demonstrated blood discharge from the nose as well as diarrhea before death. This pathology might be related to severe hemorrhage in the lung, followed by blood being carried to the nasal cavities by respiratory activities. In early reports, reovirus type 3 could induce lethal pneumonia in humans (Tillotson and Lerner, 1967). Reovirus infection also could result in lung fibrosis, acute respiratory distress syndrome (Bellum *et al.*, 1997), and bronchiolitis organizing pneumonia (London *et al.*, 2003). The newly isolated reovirus is absolutely different from those of serological type 1, type 2, and type 3 (He, *et al.*, 2003). The clinical diarrhea may be related to the large volume of water drinking to relieve high fever induced by the ReoV infection.

In histopathologic observations we determined interesting changes in different experimental infections. Animals infected with SARS-CoV solely showed less hemorrhage and hyaline-membrane formation, except for type II pneumocytic hyperplasia and protein exudation in the alveolus cavity. These changes were similar to those seen in sucking mice, cats, and ferrets (Byron *et al.*, 2003; Ron *et al.*, 2003; Thijs Kuiken *et al.*, 2003; Wang *et al.*, 2003a). With decreased titers of SARS-CoV, atypical pathological changes could be observed in the lung, spleen, and lymph nodes. This suggests that the SARS-CoV infection mainly induces fibroblast proliferation and thickness of the alveolar wall, and may not contribute to severe hemorrhagic inflammation independently. In contrast, the characteristic pathology of infection only with ReoV was mainly circulatory disturbances such as pulmonary edema, hyaline membrane formation, and hemorrhage, and cellular degeneration such as in the immune organs (spleen and lymph nodes). These changes were similar to the typical pathology at the early stages of SARS in patients (Lang *et al.*, 2003; Nicholls *et al.*, 2003; Zhao *et al.*, 2003), but the fibroblast hyperplasia and fibrosis of pulmonary tissue could not be seen. The exudation phase, proliferation, and fibrosis phases can be seen in coinfection both in Group 3 and in Group 5, which represented a three-stage process of the SARS patients (Wang *et al.*, 2003b). Our data shows that the interaction of the ReoV-cornovirus or SARS-CoV/ReoV might result in the characteristic pathology similar to the three phases of the SARS patients. It was confirmed again that ReoV might be one important pathogen in the SARS patients.

The pathogenesis of the newly isolated ReoV is largely unknown. In this experiment, a lack of neutralizing antibody activity in Groups 1 through 5 showed that the infected animals have no ability to clear the virus. As the SARS-CoV titer decreased, the antibody was gradually raised by ELISA measurement. The severe hemorrhage, necrosis, and reduction of lymphocytes in the spleen and lymph nodes also confirmed again that SARS-CoV may suppress the immunity, and aggravate the pathology of the ReoV in coinfection. Based on these findings, we proposed that pathogenesis might relate to the cellular immunity in coinfection, and SARS-CoV may predispose infected hosts to a secondary infection.

The clinical and pathological changes showed that both ReoV and SARS-CoV might replicate in the lung and immune tissues. That indicated that ReoV was a pathogen with higher virulence for lung tissue. The ReoV might bind to the virus receptors in the lung and immune tissue, leading to wide exudation, hemorrhagic inflammation, and immune system damages. On the other hand, SARS-CoV infection resulted in interstitial cell proliferation of the basement membrane of the capillary. This inflammation resulted in obstructed ventilation, anoxia, dyspnea, respiratory failure, and animal death.

Collectively, our results demonstrated that guinea pigs are susceptible to experimental infection by a newly isolated ReoV, and that this ReoV might be one of the causal agents of SARS. Based on histopathological examination of postmortem tissue, coinfection by ReoV and SARS-CoV can duplicate similar pathology of SARS and provides a useful animal model for SARS research.

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