Diffuse Alveolar Lesion in BALB/c Mice Induced with Human Reovirus BYD1 Strain and its Potential Relation with SARS

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Abstract: The objective of this study was to investigate the pathogenicity and associated lesions of a new reovirus (ReoV) isolated from patients with Severe Acute Respiratory Syndrome (SARS) in China. Twenty-five four-week-old BALB/c female mice inoculated intranasally with either ReoV (strain BYD1) alone, or ReoV combined with SARS-CoV (strain BJF) displayed ejecting fur and loss of body weight compared with control animals. ReoV and SARS-CoV were isolated from most postmortem tissues. The histopathological features of ReoV infected animals consisted of diffuse alveolar damage, with scattered hemorrhage, hyaline membrane formation and interstitial pneumonia. A typical type II pneumocyte hyperplasia and fibrogranulomatous tissue formation in the alveolar septae were observed both in the animals inoculated simultaneously with these two viruses and in the animals inoculated firstly with SARS-CoV, followed by ReoV. The animals inoculated firstly with ReoV, followed with SARS-CoV displayed scattered hemorrhage in the alveolar septa. Furthermore, other lesions in above two combination groups included depletion of lymphocytes in the germinal center of lymph nodes in the lung hilus and the spleen, hemorrhagic necrosis in white pulp of spleen, hydroid degeneration, and fatty degeneration in the liver and kidney. Mice induced with SARS-CoV alone did not display clinical signs, characteristically hyaline membrane formation, hemorrhage and early pulmonary fibrosis in lung tissue. This study demonstrated that the newly isolated ReoV might be a virulent pathogen for BALB/c mice. Mice infected firstly with SARS-CoV, followed with ReoV developed a typical diffuse alveolar lesion.

Key words: diffuse alveolar lesion, mice, reovirus, SARS-CoV

Introduction

During the period of the SARS outbreak, several agents were isolated. Mycoplasma pneumonia and some chlamydia species were suggested as possible agents in China [2, 3]. Subsequently Paramyxovirus and Metapneumovirus were suggested as possible agents of SARS in Hong Kong and in Germany [8, 9]. In March
2003, Peiris’s group identified a novel coronavirus, which had not previously been isolated from humans [8]. Although the virus was isolated from patients believed to have had SARS during the outbreak in Beijing, nearly 60 percent of patients did not have a pathogen that could be definitely identified. This would suggest that other agents or SARS-CoV in combination with other agents might have been the primary cause of SARS [15].

In March 2003, five isolates of reovirus were isolated from five SARS patients’ samples in three different labs and were adapted to a variety of cell lines [4]. Three of these five ReoV isolates were from throat swabs, and the other two were from lung tissues collected at autopsy. The reovirus suggested that the newly isolated ReoV might have been involved in the 2003 SARS outbreak, though it is not a common cause of respiratory infections, and does not often present in the respiratory tract. The new ReoVs were confirmed to have the same serotype by the unilateral cross neutralization test (designated as BYD1 strain)[6].

In order to investigate the role of this reovirus in the etiology of SARS, BALB/c mice were inoculated with ReoV alone or in combination with SARS-CoV. The clinical signs were monitored, and pathological changes evaluated to elucidate the pathogenicity of the newly isolated ReoV.

**Materials and Methods**

**Viruses, Animals and Inoculation protocol**

Reovirus was isolated from the pharynx swab of a patient with SARS and identified by Microbiology and Epidemiology Institute, Military Medical Science Academy in 2003. The isolation was passaged twice in Hep-2 cells to generate a virus stock with a titer of 10^7.5 mean tissue culture. The titer of SARS-CoV (BJF strain) was 10^4.7 TCID_{50} per 0.05 ml in Vero-6 cells, isolated and identified from the lung tissue of a patient who died of SARS in Beijing.

The current study was approved by the Animal Care and Use Committee (Academy of Military Medical Sciences, Beijing) and was carried out in an approved animal biosafety level 3 facility. Twenty-five four-week-old BALB/c female mice (Laboratory Animal Center, Academy of Military Medical Sciences, Beijing) were randomly assigned to five groups and maintained in independent ventilating cassettes (IVC) under specific pathogen-free conditions in biosafety level 3 facilities. Animals were lightly anaesthetized with an intraperitoneal (i.p.) injection of 0.08 mL of 20% ketamine and 2% acepromazine maleate (Beijing Shuanghe Medicine Company, Beijing) before inoculation. In our pilot experiment, 10^1 to 10^5 TCID_{50} of SARS-CoV were intranasally administered at a volume of 0.5 ml to six mice at each dose. Mice inoculated with 10^2 TCID_{50} of SARS-CoV developed a typical thickness of interalveolar septae. Animals were also intranasally inoculated with 10^1 to 10^{11} TCID_{50} of ReoV at the same volume. Animals inoculated with 10^2 TCID_{50} of ReoV displayed sever diffuse alveolar lesion. In the current study, group 1 mice were intranasally (i.n) infected with 0.5 ml of 10^2 TCID_{50} ReoV. Group 2 mice received simultaneously 10^2 TCID_{50} of ReoV and 10^1 TCID_{50} of SARS-CoV, and group 3 mice were inoculated firstly with 10^2 TCID_{50} of ReoV, and five days later with 10^1 TCID_{50} SARS-CoV. 10^1 TCID_{50} SARS-CoV was administered intranasally (i.n) to group 4 mice followed by 100 TCID_{50} of ReoV five days later. Group 5 mice received 10^1 TCID_{50} of SARS-CoV (Table 1). After inoculation, clinical signs were observed daily. On day 14 post-inoculation (PI), the mice were anaesthetized by lethal intraperitoneal injection with sodium pento-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Numbers</th>
<th>Virus Inoculation Schedule</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>5</td>
<td>ReoV alone</td>
<td>i.n.</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
<td>ReoV + SARS-CoV simultaneously</td>
<td>i.n.</td>
</tr>
<tr>
<td>Group 3</td>
<td>5</td>
<td>ReoV first, SARS-CoV 5 days later</td>
<td>i.n.</td>
</tr>
<tr>
<td>Group 4</td>
<td>5</td>
<td>SARS-CoV first, ReoV 5 days later</td>
<td>i.n.</td>
</tr>
<tr>
<td>Group 5</td>
<td>5</td>
<td>SARS-CoV alone</td>
<td>i.n.</td>
</tr>
</tbody>
</table>

*: mice numbers in the group. #: intranasal administration.
barbital and sacrificed. The gross lesions were recorded and the main organs including the lung, kidney, liver and spleen were collected and each divided into two sub-samples. One sub-sample from each group was stored at −70°C for virus isolation and the other was fixed in 10% neutral buffered formalin for histopathology.

**Virus isolation and identification**

From each group, tissues from three mice were randomly chosen and frozen-thawed three times in 2 ml of 0.5% gelatin dissolved in phosphate-buffered saline (pH =7.2), then homogenized using tissue grinders and a Virsonic 475 sonicator. After centrifugation at 3,000 rpm for 20 min, 1 ml of the resultant supernatant was used to inoculate Hep-2 cells and Vero-6 cells, respectively. The isolated virus was identified by observation of the typical cytopathological effect (CPE) and by RT-PCR amplification using supernatants [4].

**Histopathology**

The remaining sub-samples of lung, spleen, liver and kidney were placed in 10% buffered formalin, paraffin embedded, sectioned at 5 µm and routinely stained with hematoxylin-eosin for histopathological assessment.

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**Results**

**Clinical signs**

The mice inoculated with ReoV alone (group 1) demonstrated poor appetites and developed lower body weight gain at day 14 PI (Fig. 1), compared with mice infected by SARS-CoV alone (group 5), while the mice simultaneously inoculated with ReoV and SARS-CoV (group 2) and inoculated with ReoV and then SARS-CoV (group 3) did not show the above symptoms. In the current study, mice infected with SARS-CoV, followed 5 days later with ReoV (group 4) displayed dorsiflexion, unkempt fur and unbalanced walking (Fig. 2). Furthermore, these mice also showed lower average body weight gain at the end of experiment. Most of the mice in the 5 groups survived during the observation period (Table 2).

**Virus isolation**

ReoV and SARS-CoV were isolated from the lung, liver and spleen (Table 3). In mice infected with ReoV, SARS-CoV, or both ReoV and SARS-CoV, the infect-

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Fig. 1. (A) Mice infected with SARS-CoV showed no clinical signs on day 14 postinfection. (B) Mice inoculated with ReoV BYD1 displayed lower body weight.

Fig. 2. (A) Mouse infected with SARS-CoV alone was normal on clinical inspection on day 7 postinfection. (B) Mice inoculated firstly with SARS-CoV, then with ReoV showed unkempt fur.
ing agents were in lung and liver samples with higher positive isolation ratios than in kidney and spleen samples. The highest positive isolation ratios for ReoV and SARS were found in group 4, which corresponded with the clinical symptoms.

The gross and microscopic examination

At necropsy, there were no evident changes of the liver, spleen and heart, except for extensive consolidation of the lungs, in group 1 and group 4 animals, which displayed a red color on the surface and had focal hemorrhages. There was some pink to red fluid when the lungs were removed and a small amount of secretion in the respiratory tract was observed both in group 1 and group 4 animals (Table 4). The characteristic changes of lungs infected with ReoV alone showed wide hemorrhage, protein exudation and hyaline membrane formation in the alveolar septae, together with a few active proliferation of type II pneumocytes (Fig. 3). The main pathological changes in group 2 mice (coinfected simultaneously with ReoV and SARS-CoV) were acute pulmonary interstitial exudation, diffuse hemorrhage (Fig. 4) and proliferation of type II pneumocytes in the interalveolar space (Fig. 5). In group 3 mice, there was mild leakage inflammation, including local hemorrhage in the interalveolar spaces and less fibrotic inflammation (Fig. 6). The pathological changes in the group 4 were more severe than those in group 1, group 2 and group 3. Almost 60% of the lungs displayed interstitial pneumonia, characterized by hyaline membrane formation (Fig. 7), intraalveolar edema, type II pneumocytes hyperplasia, microthrombi, and early fibrosis of pulmonary tissue (Fig. 8). Multinuclear syncytoid cells were also found in the alveolar spaces. In contrast, mice infected by SARS-CoV developed a thickness of interalveolar septae due to the proliferation of the type II alveolar cells and erythrocytes leakage. Another typical change was less hyaline membrane formation, hemorrhage and no early fibrosis of pulmonary tissue (Fig. 9).

The normal structure of lymph nodes at the hilus of the lung disappeared with evident expansion of the lymphatic sinus and vessels both in the ReoV alone inoculation group and in the groups co-infected with SARS-CoV. The lymphocyte numbers in the cortices were greatly reduced and the size of the germinal center in white pulp decreased (Fig. 10). Focal necrotic inflammation occurred in the lymph nodes at the hilus

Table 2. Clinical signs and mortality occurring in the treatment groups after inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g) p.i.</th>
<th>Clinical signs</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>Group 1</td>
<td>20.3 ± 0.5</td>
<td>18.1 ± 1.1a</td>
<td>13.7 ± 1.3a</td>
</tr>
<tr>
<td>Group 2</td>
<td>19.5 ± 0.3</td>
<td>20.1 ± 1.5b</td>
<td>19.5 ± 1.1b</td>
</tr>
<tr>
<td>Group 3</td>
<td>18.98 ± 0.6</td>
<td>19.7 ± 1.0b</td>
<td>20.1 ± 1.0b</td>
</tr>
<tr>
<td>Group 4</td>
<td>20.0 ± 0.8</td>
<td>19.2 ± 1.8b</td>
<td>11.2 ± 1.3b</td>
</tr>
<tr>
<td>Group 5</td>
<td>19.2 ± 0.8</td>
<td>20.5 ± 1.7b</td>
<td>21.2 ± 1.2b</td>
</tr>
</tbody>
</table>

a, b: Different superscripts in a row indicate statistical significance (P<0.05).

Table 3. Isolated ReoV and SARS-CoV in different tissues at day 14 postinfection

<table>
<thead>
<tr>
<th>Organs</th>
<th>No.positive/totalb</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReoV</td>
<td>SARS-CoV</td>
<td>ReoV</td>
<td>SARS-CoV</td>
<td>ReoV</td>
<td>SARS-CoV</td>
</tr>
<tr>
<td>Group 1</td>
<td>3/3</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Group 2</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Group 3</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Group 4</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Group 5</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

< Number of mice in which the virus was detected in the indicated organs out of total number of mice evaluated.
of the lung. There were ecchymoma in the central artery and venule. There was myocardial stromal edema in cardiac muscle fiber, in which scattered hemorrhage was also evident. Moreover, hepatocytes had fatty degeneration, cloudy swelling, apoptosis, and dot necrosis, with Kupffer cell proliferation and portal infiltrates of lymphocytes both in group 1 and group 4.

Discussion

In the current experiment, the animals inoculated with ReoV alone and in combination with SARS-CoV showed obvious clinical signs and typical hyaline membrane formation in the lung, although no morbid case occurred. ReoV was identified in both the ReoV-infected mice and in the co-infected mice. In a pilot study, *macaques* infected by human ReoV isolates developed multiple foci of pulmonary consolidation in bilateral lungs, and patchy fibrosis in the walls of the alveoli [7]. The results of the present investigation of ReoV-infected BALB/c met Koch’s postulates about establishing a virus pathogen: isolation of the virus from diseased hosts, cultivation in cells, proof of filterability, duplication of a comparable pathology in original or related species and re-isolation of the virus from experimentally diseased hosts [5]. The results of the present study suggest that the newly isolated ReoV may be a virulent pathogen in BALB/c mice.

Pathogenicity of ReoV in humans has not attracted much attention for a long time. There are no reports concerning ReoV isolated from respiratory tract samples in China before 2003, though reovirus 1/L and reovirus 3/D caused acute pneumonia in SD rats, marked by type I alveolar epithelial cell degeneration, type II alveolar epithelial cell hyperplasia and infiltration of leukocytes [10]. Reovirus 1/L induced acute respiratory distress syndrome (ARDS) and bronchiolitis obliterans organizing pneumonia (BOOP) both in BALB/c mice and *CBA/J* mice [1, 11]. In this study, the BALB/c mice infected with ReoV alone developed an exudative pneumonia and extensive hemorrhagic necrosis in lymph nodes and the spleen. This pattern of pathology without the involvement of intraluminal fibrosis is not consistent with the infection of reovirus 1/L in BALB/c mice [14]. These histological results indicate that this newly isolated reovirus is different from the formerly reported reovirus 1/L, which was correlated with a previous serotype identification result [6]. In the mixed inoculation groups, wide hemorrhage and proliferating type II pneumocytes in the lung were the most notable features, but fibroblastic proliferation in the alveolar septa, which leads to obliteration of the alveolar space and pulmonary fibrosis was observed in the animals inoculated firstly with SARS-CoV, followed by ReoV. These histopathological lesions are analogous to those described in humans in the late course of SARS [9, 17, 18]: proliferation of fibroblasts and deposition of collagen fibrin in the interstitia, leading to

<table>
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<th>Gross lesions</th>
<th>Histopathological change</th>
<th>Other findings</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Hyaline membrane formation</td>
<td>Interstitial pneumonitis*</td>
</tr>
<tr>
<td>Group 1</td>
<td>Bilateral consolidation</td>
<td>++++</td>
<td>Fibrin, RBC, type II pneumocyte</td>
</tr>
<tr>
<td>Group 2</td>
<td>Focal hemorrhage</td>
<td>++</td>
<td>type II pneumocyte, RBC and fibroblast</td>
</tr>
<tr>
<td>Group 3</td>
<td>Hemorrhage</td>
<td>++</td>
<td>RBC, fibrin</td>
</tr>
<tr>
<td>Group 4</td>
<td>Extensive bilateral consolidation</td>
<td>+++</td>
<td>RBC, fibroblast, type II, fibrin</td>
</tr>
<tr>
<td>Group 5</td>
<td>Focal hemorrhage</td>
<td>_</td>
<td>type II pneumocyte, fibrin</td>
</tr>
</tbody>
</table>

+++ Indicates the extent of hyaline membrane formation.
* Interstitial infiltration consisted of different inflammatory cells after virus inoculation.
Fig. 3. Histopathological change in a lung section from a mouse infected with ReoV. Hematoxylin & eosin staining. ReoV infection induced severe hyaline membrane formations (arrow) and fibrinous deposition.

Fig. 4. Morphological changes in a lung section from a mouse infected with ReoV and SARS-CoV. Hematoxylin & eosin staining. Focal intra-alveolar hemorrhage (arrow) and intravascular thrombosis were observed in the lung.

Fig. 5. Microscopy of lung section from a mouse inoculated simultaneously with ReoV and SARS-CoV. Hematoxylin & eosin staining. The alveolar spaces were filled with fibrinous exudates and scattered type II pneumocytes (arrow).

Fig. 6. Histological lesions in a lung from a mouse inoculated firstly with ReoV, followed by SARS-CoV. Hematoxylin & eosin staining. Scattered hemorrhage was seen in the alveolar septa (arrow).
Fig. 7. Histological lesions in lungs from a mouse infected with SARS-CoV, followed by ReoV. Hematoxylin & eosin staining. Exudative fluid containing fibrin and epithelial cells was observed together with light red fibrinous deposition on alveolar walls and hyaline membrane formation (arrow) in alveolar spaces.

Fig. 8. Histological lesions in the lung of a mouse infected with SARS-CoV, followed by ReoV. Hematoxylin & eosin staining. The entire lung showed fibroblastic proliferation in alveolar spaces (arrow).

Fig. 9. Histological lesions in the lung of a mouse inoculated with SARS-CoV. Hematoxylin & eosin staining. Mild interstitial lesions were observed in the lungs due to inflammatory infiltrates (arrow).

Fig. 10. Morphological change in a spleen section from a mouse inoculated with SARS-CoV, then with ReoV five days later. Hematoxylin & eosin staining. Hemorrhagic necrotic inflammation was observed near the capsule of the spleen (arrow).
early fibrosis of pulmonary tissue. In addition, group 4 (SARS-CoV and then ReoV) showed more severe symptoms and histopathological lesions than group 3 (ReoV and then SARS-CoV), group 2 (simultaneously inoculated) and group 5 (SARS-CoV alone) mice. This implies that the development of fibrosis was dependent on the deposition of fibrin in the alveoli and the order of inoculation of the virus strain. The onset of fibrosis is a critical feature of chronic diffuse alveolar damage, because it leads to loss of alveolar function and is irreversible. In contrast to the lung lesions observed after inoculation with SARS-CoV alone, there was mild lesion in both the alveolar septa and in the immune system in group 5 mice, a results which agrees with previous reports on KM sucking mice, BALB/c mice, golden Syrian hamsters, cats, ferrets and cynomologus macaques [2, 8, 12, 13, 16, 18]. These infected animals displayed the atypical pathology of the second and third phases of human SARS patients [2, 8, 12, 13]. SARS-CoV infection suppresses immunity and may predispose infected hosts to secondary infections, such as measles virus infection [8]. In consistently infected animals, SARS-CoV may play a role in the proliferation of type II pneumocytes and early pulmonary fibrosis where it may induce proliferation of type II pneumocytes and organizing pneumonia in co-infected animals. After infection with SARS-CoV, ReoV induced severe alveolar lesions and hyaline membrane formation resulting in low body weight and unkempt fur. In this study, the target organs of infection included the lung, lymph nodes and spleen. Pneumocytes appeared to be the most sensitive target cells. ReoV and SARS-CoV induced severe damage in the alveolar epithelial and capillary endothelial cells, leading to pulmonary edema, intra-alveolar deposits and alveolar fibrosis. In addition to respiratory system pathology, damage to the immune system occurred whether the animals were infected with ReoV or infected with both ReoV and SARS-CoV. This damage was characterized by focal necrotic inflammation near the capsule of the spleen. All of this suggests that ReoV and SARS-CoV might replicate in the pulmonary organ and stimulate the immune system, leading to severe immunological damage. Based on these findings, together with immunity suppression observed in cynomologus macaques [7], we speculate that SARS-CoV may aggravate ReoV infection leading to pulmonary fibrosis in the lung and other typical pathological lesions in extra pulmonary organs.

This study demonstrated that ReoV is a virulent pathogen for BALB/c mice, and that the interaction of SARS-CoV and ReoV can induce the typical diffuse alveolar damage described in SARS cases as it progresses from the initial stages of exudation to fibrotic lesions developing in the later stages. Further investigation is needed to describe the newly isolated ReoV’s role in the pathogenesis of SARS.

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References


