Respiratory syncytial virus nephropathy in rats

X-M Liu1,2, Z Wang1 and Y Guo1

1Department of Pediatrics, Second Hospital of West China, Sichuan University, Chengdu, China; 2Department of Medicine, Xuzhou Children’s Hospital, Xuzhou, China

The pathogenesis of minimal change nephrotic syndrome (MCNS) remains unclear. Respiratory tract viruses could contribute to MCNS, and respiratory syncytial virus (RSV) is the most common one. In this study, we planned to investigate the effects of RSV on the proteinuria and glomerular structure of rats and to explore the role of RSV in the pathogenesis of MCNS. Rats were inoculated with 6 × 102, 104, and 106 PFU (plaque-forming units) RSV and killed on days 4, 8, 14, 28, and 60 postinoculation (RSV4R, RSV8R, RSV14R, RSV28R, and RSV60R). The proteinuria and serum parameters were measured; renal histology was observed by light microscopy and electron microscopy; immune complex deposits were detected by immunofluorescence microscopy; and RSV RNA and RSV titer were determined by in situ hybridization and plaque assay, respectively. After inoculation, the proteinuria increased, especially in 6 × 106 PFU RSV14R and RSV28R and different-titer RSV60R decreased. Slight hypercellularity in minority glomeruli and swelling in partial tubular epithelial cells were observed in RSV4R, whereas a relief of the above changes and no abnormalities were detected in RSV14R and RSV28R60R, respectively, under a light microscope. Extensive foot process effacement was observed in 6 × 106 PFU RSV14R28R60R under an electron microscope. No immune complex deposits were detected in the renal tissues. RSV RNA signal and RSV titer of renal tissues, depending on the dose of inoculum, reached their climax on day 8 postinoculation. Our study reports for the first time that RSV can lead to nephropathy in rats on days 14-60 postinoculation, especially in 6 × 106 PFU RSV-inoculated rats, which may be a new exploration of the pathogenesis of MCNS.


KEYWORDS: respiratory syncytial virus; proteinuria; foot process; nephropathy; animal model

Correspondence: Z Wang, Department of Pediatrics, Second Hospital of West China, Sichuan University, No. 20, Renmin NanLu 3rd Section, Chengdu 610041, China. E-mail: Wangzheng48@21cn.com

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manner. With different titers, we inoculated rats and intended to study the effects of RSV on the proteinuria and serum albumin, cholesterol and glomerular structure of rats at different inoculation time. We detected RSV in renal and pulmonary tissues by in situ hybridization and plaque assay to explore the construction of the RSV nephropathy model and the role of RSV in MCNS.

RESULTS
Proteinuria excretion
After inoculation, the proteinuria excretion of rats inoculated with different-titer RSV increased gradually and reached their climax on days 14–18 postinoculation. Excretion remained increased even on day 28 and day 60 postinoculation, especially in $6 \times 10^6$PFU (plaque-forming units) groups (Figure 1). The proteinuria excretion of $6 \times 10^6$PFU RSV (14,28,60) were much higher than those of $6 \times 10^2$PFU RSV14,28,60 of CB3, Dulbecco’s modified Eagle’s medium (DMEM), and normal control and the group before inoculation, $P<0.05$. However, no significant differences were found either between $6 \times 10^5$ and $6 \times 10^6$PFU RSV-inoculated groups or among different time points of days 4, 8, 14, 28, and 60 of CB3, DMEM, and normal control groups, $P>0.05$ (Table 1).

Serum parameters
At 4 and 8 days after RSV inoculation, no significant differences of serum albumin and cholesterol were detected among different-titer groups. Although urea nitrogen and creatinine of the individual increased, there was no statistical significance among all groups (including RSV-inoculated groups and all control groups). At 14 and 28 days after RSV inoculation, serum albumin of $6 \times 10^6$PFU RSV 14 (15.06 ± 1.34 g/l) and $6 \times 10^6$PFU RSV 28 (16.95 ± 1.43 g/l) were lower compared with control groups of CB3 (19.04 ± 1.58 g/l), CB3 28 (18.33 ± 2.27 g/l), DMEM (18.73 ± 0.84 g/l), DMEM28 (20.53 ± 1.77 g/l), Normal 14 (19.98 ± 1.06 g/l), and Normal28 (21.78 ± 3.85 g/l), and 60 days after RSV inoculation, serum albumin of $6 \times 10^6$PFU RSV 14 (20.85 ± 1.50 g/l), $6 \times 10^4$ (20.23 ± 1.24 g/l), and $6 \times 10^2$ (21.17 ± 0.91 g/l) PFU RSV 60 were still lower compared with normal controls (24.43 ± 1.53 g/l), $P<0.05$. However, there were no statistical differences of serum cholesterol, urea nitrogen, and creatinine among all groups.

Histopathological findings in the kidney and lung
Macroscopic findings. Partial kidneys of $6 \times 10^5$, $6 \times 10^4$, and $6 \times 10^2$PFU RSV8/14 were swollen and grains were presented on the surfaces of the minority; the others were normal.

Under a light microscope. Slight hypercellularity in minority glomeruli (Figure 2a), swelling and vascular degeneration in partial tubular epithelial cells, together with slight inflammatory cell infiltration in the interstitium (Figure 2b) were observed in $6 \times 10^5$, $6 \times 10^4$, and $6 \times 10^2$PFU RSV, whereas a relief of the above changes and no abnormalities were detected in RSV14 (Figure 2c) and RSV28/60 (Figure 2d–e), respectively. Inflammation observed in the lungs of RSV4 was associated with inflammatory cell infiltration and edema of the bronchiolar wall and peribronchiolar areas. The most significant changes were observed in the lungs of RSV8, especially in the $6 \times 10^6$PFU RSV-inoculated group. There was considerable inflammatory cell infiltration of alveolar wall and terminal

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Quantitative analysis of proteinuria excretion</th>
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<tr>
<td>Postinoculation time (d)</td>
<td>RSV titer (PFU)</td>
</tr>
<tr>
<td>0</td>
<td>4.46 ± 2.17</td>
</tr>
<tr>
<td>4</td>
<td>11.97 ± 4.19*</td>
</tr>
<tr>
<td>8</td>
<td>17.95 ± 4.47*</td>
</tr>
<tr>
<td>14</td>
<td>32.04 ± 3.84*</td>
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<tr>
<td>28</td>
<td>31.69 ± 2.07*</td>
</tr>
<tr>
<td>60</td>
<td>20.68 ± 8.66*</td>
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DMEM, Dulbecco’s modified Eagle’s medium; PFU, plaque-forming unit.

Date are presented as means ± s.d. *P < 0.05 vs CB3, DMEM control and normal control;
$^o$P < 0.05 vs $6 \times 10^5$, $6 \times 10^4$ PFU;
RSV-inoculated groups; $^o$P < 0.05 vs RSV4 and RSV8 at the same RSV titer.
peribronchiolar areas. The interalveolar septa were thickened obviously. A less marked interstitial inflammation and no inflammation were presented on the lungs of RSV14, RSV28, and RSV60, respectively. Interstitial inflammation and thickening of the interalveolar septa were observed in the CB3 control group, but it was less compared with the $6 \times 10^6$ PFU RSV-inoculated groups. No abnormalities were observed in the kidneys and lungs of rats in DMEM and normal control groups.

**Under an electron microscope.** The main changes in kidneys of $6 \times 10^6$, $6 \times 10^5$, and $6 \times 10^2$ PFU RSV were swelling and effacement of foot processes of glomerular epithelial cells, and there was obvious aggravation in $6 \times 10^6$ PFU RSV-inoculated groups when compared with $6 \times 10^4$ and $6 \times 10^2$ PFU RSV-inoculated groups. Foot process effacement of $6 \times 10^6$ PFU RSV1428 (Figure 3b and c) was conspicuous and it was extensive and generalized in $6 \times 10^6$ PFU RSV60 (Figure 3d). Partial glomeruli of $6 \times 10^6$, $6 \times 10^5$, and $6 \times 10^2$ PFU RSV1428 exhibited chromatin margination, chondriosome swelling, and slight hyperplasia in endothelial cells and mesangial cells. Besides, no electron-dense deposit and separation of foot process and glomerular basement membrane were found in different-titer RSV-inoculated groups and only slight hyperplasia in mesangial cells was found occasionally in $6 \times 10^6$, $6 \times 10^5$, and $6 \times 10^2$ PFU RSV60. The glomeruli of all control groups showed normal foot processes and structures (Figure 3g-i).

**Immunofluorescence detection of IgG, IgA, IgM, and C3 deposits.** No immunofluorescence of IgG, IgM, IgA, and C3 deposits was detected in the renal tissues of rats in $6 \times 10^6$ PFU RSV60 (Figure 4a-d) and normal control by immunofluorescence microscopy.

**Detection of RSV RNA in renal and pulmonary tissues by in situ hybridization.** Figure 5 shows that the dark brown grains, indicating a positive hybridization signal, appeared in the epithelial cells and mesangial cells of glomeruli and tubular epithelial cells (Figure 5b–d) in $6 \times 10^6$ and $6 \times 10^5$ PFU RSV1428. The bronchiolar epithelial cells and pulmonary alveoli of the pulmonary tissues of $6 \times 10^6$, $6 \times 10^5$ PFU RSV1428 and $6 \times 10^2$ PFU RSV48 also showed a positive signal. No positive signal could be detected in $6 \times 10^6$ PFU RSV2860 (Figure 5e and f), $6 \times 10^4$ PFU RSV2860, $6 \times 10^2$ PFU RSV-inoculated groups and all control groups. RSV-inoculated HeLa cells served as a positive control (Figure 5a) and integral optical density semiquantitative analysis of positive signal in renal tissue was performed. The signal of $6 \times 10^6$ PFU RSV8 was stronger than those seen in other groups ($P<0.05$) (Table 2).

**RSV titers in renal and pulmonary tissues of RSV-inoculated rats.** Viral titers in the renal and pulmonary tissues of rats inoculated with different-titer RSV were measured by plaque assay. Figure 6 illustrates that RSV titers (expressed with PFU) in the renal and pulmonary tissues, depending on the dose of viral inoculum, reached their climax on day 8 postinoculation ($6 \times 10^5$ PFU RSV4, $3.89 \pm 0.16$, $6 \times 10^5$ PFU RSV8, $2.20 \pm 0.28 \log_{10}$ PFU/g renal tissue, and $6 \times 10^6$ PFU RSV8, $5.21 \pm 0.21$, $6 \times 10^6$ PFU RSV8, $3.90 \pm 0.16$, $6 \times 10^6$ PFU RSV8, $1.65 \pm 0.16 \log_{10}$ PFU/g pulmonary tissue). From day 8 onwards, the PFU decreased gradually ($6 \times 10^5$ PFU RSV14, $2.00 \pm 0.14$, $6 \times 10^4$ PFU RSV14, $1.10 \pm 0.17 \log_{10}$ PFU/g renal tissue and $6 \times 10^5$ PFU RSV14, $2.97 \pm 0.07$, $6 \times 10^4$ PFU RSV14, $1.40 \pm 0.17 \log_{10}$ PFU/g pulmonary tissue), and no plaque forming was detectable in the renal tissues of different-titer RSV2860. $6 \times 10^2$ PFU RSV-inoculated groups and all control groups. RSV titers in the renal and pulmonary tissues of $6 \times 10^6$ PFU RSV8 were higher than those of $6 \times 10^6$ PFU RSV142860.

**Relative kidney weight, body weight, and general state of health.** Relative kidney weight was expressed as kidney weight/body weight. Statistical analysis showed that $P<0.05$ when the relative kidney weight of different-titer RSV8 ($6 \times 10^5$ PFU: $66.08 \times 10^{-4} - 10.5 \times 10^{-4}$, $6 \times 10^4$ PFU: $58.35 \times 10^{-4} - 5.28 \times 10^{-4}$, $6 \times 10^2$ PFU: $61.34 \times 10^{-4} - 6.78 \times 10^{-4}$) was compared with those of all control groups (CB38: $46.33 \times 10^{-4}$ $- 4.01 \times 10^{-4}$, DMEM: $44.75 \times 10^{-4}$ $- 4.55 \times 10^{-4}$, Normal: $46.74 \times 10^{-4}$ $- 3.62 \times 10^{-4}$). However, there was no statistical
difference among different-titer RSV 4,14,28,60 and all control groups. RSV inoculation resulted in reduced weight gain compared to DMEM and normal control. Rats inoculated with RSV developed different degrees of depression, appetite and activity reduction, hair dropping, and fur wrinkling. Anabrosis of nasal mucosa and a slight increase in nasal discharge, increased respiratory rate (mostly presented on 5-6 days after inoculation), temporary hematuria (mostly presented on days 2–8 postinoculation and disappeared within 14 days), and increased crystals of centrifuged urine were observed in some rats. Light yellow and lucid hydrogaster was discovered in a rat of $6 \times 10^2$ PFU RSV 14, whereas no pleural fluid, hydrogaster, and scrotal edema were discovered in the rats of other groups. During experimental session, rats of DMEM and normal control developed no abnormal appearance, whereas a few CB3 controls developed increased respiratory rate. In addition, one of the CB3 controls developed paralysis of the lower left limb and lameness, which was similar to manifestations of poliomyelitis, and then recovered without any treatment.

Figure 3 | Ultrastructure of the renal tissues of RSV-inoculated rats under an electron microscope. (a) Effacement of partial foot processes was presented in $6 \times 10^6$ PFU RSV 4. Foot process effacement was extensive in (b) $6 \times 10^6$ PFU RSV 14, (c) RSV 28, and (d) RSV 60. Effacement of partial foot processes was presented in (e) $6 \times 10^7$ and (f) $6 \times 10^8$ PFU RSV 14. Normal and clear foot processes were presented in (g) CB3, (h) DMEM, and (i) normal control groups. The arrows indicate foot process effacement. Original magnification (a, f) $\times 12000$, (b, e) $\times 5000$, (c) $\times 15000$, (g, i) $\times 8000$, and (d, h) $\times 10000$. 
DISCUSSION

RSV is an important and common viral pathogen that produces worldwide lower respiratory infection not only in infants and young children but also in the aged and adults with immunosuppression.\textsuperscript{9–11} At present, studies on RSV mainly focus on the mechanism of respiratory tract infection, generally considering that RSV infection often occurs in the respiratory tract and spreads from the upper respiratory tract to the lower respiratory tract by cell–cell transfer. Recently, however, it was reported that RSV infection had a viremia and could invade other organs and tissues. For instance, by reverse transcriptase-polymerase chain reaction, Valentova,\textsuperscript{12} Yui,\textsuperscript{13} and O’Donnell\textsuperscript{14} detected RSV RNA in the peripheral blood leukocytes of natural infected cattle and the peripheral blood cells of lower respiratory tract infected patients, respectively. Keles\textsuperscript{15} and Sharma\textsuperscript{16} also found that bovine RSV could replicate in bovine and ovine peripheral blood lymphocytes and monocytic cell lines. Furthermore, our early studies found that RSV and its antibody were present not only in the epithelial cells of the respiratory tract but also in the PBMC, serum, urine, and renal tissue of SRNS,\textsuperscript{3–5} suggesting that RSV might be a contributor to the onset and development of SRNS.

In our present study, we intended to observe proteinuria excretion and damage to the kidneys of rats inoculated with RSV and to explore the effects and mechanisms of RSV in MCNS. The results of our study showed that, on days 4–8 postinoculation, (1) proteinuria excretion increased gradually, (2) no significant changes were found in serum albumin and cholesterol, (3) under a light microscope, slight hypercellularity in minority glomeruli, swelling and vacuolar degeneration in partial tubular epithelial cells, together with slight inflammatory cell infiltration in the renal interstitium and interstitial pneumonia-like change in the lung were observed, which were all most significant on the 8th day postinoculation, (4) swelling and effacement of partial foot processes of glomerular epithelial cells were observed under an electron microscope, and (5) both RSV RNA and higher titers of RSV were detected in the renal and pulmonary tissues of rats inoculated with $6 \times 10^6$ and $6 \times 10^4$ PFU RSV and they both reached their climax on day 8 postinoculation, which coincided with pathological changes under a light microscope and further suggested that RSV infection existed in renal tissue.

Our findings on days 14–60 postinoculation were as follows: (1) 14 days after inoculation, maximal excretion of proteinuria of rats was detected, especially in rats inoculated with $6 \times 10^6$ PFU RSV, and the excretion maintained a high level even on day 60 postinoculation; (2) the serum albumin of $6 \times 10^6$, $6 \times 10^5$, $6 \times 10^4$, and $6 \times 10^2$ PFU RSV decreased; (3) relief of the pathological changes in glomeruli, tubules, and renal interstitium and an interstitial pneumonia-like change in lungs were observed on day 14 postinoculation; (4) foot process effacement was conspicuous on day 14 postinoculation and it was more extensive and generalized on day 60 postinoculation, especially in $6 \times 10^6$ PFU RSV-inoculated rats; (5) on day 60 postinoculation, no immune complex deposits were observed in glomeruli; (6) both the positive signals of RSV.
RNA and RSV titers showed an apparent decrease on day 14, and until day 28 and day 60 postinoculation when they were not detected at all.

According to the above results, the damage of RSV to the rat’s kidney occurs in two stages. In the first stage (on days 4–8 postinoculation), the damage to the kidney was mainly caused by direct inflammatory reaction of RSV, with its multiple possible mechanisms listed as follows: RSV infection could lead to increasing degranulation of the peripheral blood neutrophils and release of some inflammatory enzymes such as elastase and heparanase; RSV infection induced the activation of a number of cytokines and chemokines, which could damage the glomerular filtration barrier directly by itself. Our early studies have revealed an increased expression of heparanase mRNA in peripheral blood leukocytes of SRNS, and the expression of heparanase mRNA was positively relevant to proteinuria excretion. In our present study, signals of RSV RNA were detected in glomerular epithelial cells by in situ hybridization. All these results laid the groundwork for the above-possible mechanisms. Does this direct inflammatory reaction of virus also cause damage to the kidneys of patients with MCNS? The previous studies suggested this possibility, for they already showed respiratory tract viruses in diverse tissues and cells, including the urine and renal tissue of patients with SRNS. In another study, we worked on the change of elastase in the peripheral blood of children with SRNS and rats infected by RSV and the mechanism of RSV’s direct damage to the kidney.

In the second stage (on days 14–60 postinoculation), the pathological changes under a light microscope became less or disappeared, the pathogen of RSV in renal tissue decreased or was even nonexistent, and no immune complexes were found in the glomeruli. Nevertheless, proteinuria excretion and foot process effacement were more obvious and accompanied hypoalbuminemia, which were similar to the manifestation of human McNS. Then, what is the fundamental mechanism of proteinuria and changes in the kidney at this stage? It still remains poorly understood, and inflammation by direct infection may not be responsible. It could be related to the impairment of virus in the immune system and disorder of immunoregulation.

Because proteinuria excretion increased after RSV inoculation, and was still much higher than that of normal control even on day 60 postinoculation, we suggest that the proteinuria caused by RSV inoculation was pathological proteinuria rather than transient febrile proteinuria. The reason why proteinuria caused by RSV inoculation was not as notable as that caused by puromycin or Adriamycin may be due to different mechanisms of developing renal lesions and proteinuria. Moreover, the changes caused by RSV in the second stage of our present study were similar to human MCNS except for hypercholesterolemia. Are there species differences between rats and humans? Also, whether there is a direct causality between RSV and MCNS needs to be studied further to provide potent evidence for data in the present study. Nevertheless, our present study suggests that RSV could cause nephropathy in rats, including multiple possible mechanisms such as direct damage to the kidney and the disorder of immunological response, plus the effects of some enzymes from viral infection. Although abnormal immunological response after virus infection cannot be excluded, direct damage from the proper virus infection should not be neglected. We are conducting further study on this to make clear the problem and will report it in another paper.

In addition, in our present study, rats inoculated with $6 \times 10^6$ PFU CB3 served as control, in which although symptoms of respiratory tract and nervous system developed, no significant increase of proteinuria excretion and pathological change in kidney were detected. The reasons responsible for the above phenomena may be the following: (1) Although CB3 can cause inflammatory reaction, it has no kidney tissue affinity, which is determined by co-action...
between viral binding protein and specific receptors of the cell surface.\(^2\) Without enough strong positive charge, CB3 is unable to have real binding with the negative charge in glomerular cells and the filtration barrier through electrostatic attraction so as to damage the glomeruli directly.

To our knowledge, our present study showed for the first time that the proteinuria excretion of rats inoculated with RSV increased, the serum albumin decreased, and effacement of foot processes of glomerular epithelial cells was extensive and generalized, particularly in rats inoculated with \(6 \times 10^8\) PFU RSV on days 14–60 postinoculation. The RSV nephropathy rat model we set up is advantageous for further study on the correlation between respiratory tract viruses and glomerular diseases. It has provided strong evidence for the fact that respiratory tract viruses play an important role in the onset and development of MCNS, thus giving impetus for further exploration of the pathogenesis of MCNS.

MATERIALS AND METHODS

**Virus and cells**

The long strain of human RSV and HeLa cell strain were purchased from the viral institute of Chinese Academy of Preventive Medical Science. HeLa cells were grown in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) at 37°C in 5% CO\(_2\) and RSV was propagated in HeLa cells in a monolayer culture. At 3 or 4 days after RSV was incubated with HeLa cells, when cell fusion was most obvious, the culture flask was shaken vigorously to make cells fall off from the wall of the flask. Stocks of RSV were prepared by three freeze–thaw cycles, followed by centrifugation at 2000 \(g\) (4°C) for 10 min to remove cell debris and remain as supernatant. Stocks were aliquoted, quick frozen, and stored at \(-70^\circ\)C until use. The RSV titer was determined by a methylcellulose plaque assay.

**Animals**

Five- or six-week-old specific pathogen-free Sprague–Dawley male rats weighing 150–200 g were supplied by West China Medical Animal Center (Sichuan University, China) and maintained in a pathogen-free environment, in accordance with the guidelines in the National Institutes Health Guide for the Care and Use of Laboratory Animals. Each rat was kept in an isolated metabolic cage. Separate rooms were used to breed experimental and control rats, both of which were fed with sterilized food and water.

**Inoculation of animals**

The rats were divided randomly into groups for 4, 8, 14, 28, and 60 days after inoculation (RSV4, RSV8, RSV14, RSV28, and RSV60), and each of the five rats in different groups was inoculated intranasally (0.2 ml) and intraperitoneally (0.4 ml) with \(6 \times 10^4\) and \(6 \times 10^5\) PFU RSV diluted in DMEM, respectively. Once a day up to 3 days, control rats were inoculated with the same volume of either \(6 \times 10^5\) PFU CB3 or virus-free DMEM or nothing (normal control) in the same way. The fur, behavior, appetite, respiration, weight of the rats, and the aspect of urine were monitored.

**Preparation of samples**

**General samples.** 5 ml aliquot of the 24-h urine collected by metabolic cage was used. The blood samples were collected from the orbital veniplex of the rats killed on days 4, 8, 14, 28, and 60 postinoculation.

**Renal and pulmonary samples.** Under anesthesia by intraperitoneal injection of 10% chloral hydrate and sterile conditions, the right kidneys and lungs of rats were resected, washed with 0.9% sodium chloride, and weighed on an electronic balance (the renal envelope and connective tissue of renal hilum were removed). Flakes from renal tissues were fixed immediately in 3% glutaraldehyde buffer for electron microscope analysis, and the remaining tissues and right lungs were used for immunofluorescence studies and viral plaque assay. General circulation perfusion was then performed by 0.9% sodium chloride and 4% paraformaldehyde containing 1% diethylpyrocarbonate (Sigma, St Louis, MO, USA) in sequence. 0.5 cm \(\times\) 0.75 cm tissues from the left kidneys and lungs were refixed in 4% paraformaldehyde at 4°C for another 24 h and processed by routine paraffin-embedding methods for histopathological analysis and \textit{in situ} hybridization.

**Measurement of proteinuria and serum parameters**

Proteinuria excretion was measured by the pyrogallol endpoint method, and serum albumin, cholesterol, urea nitrogen, and creatinine by an automatic biochemical instrument (Hitachi 7600, Japan).

**Histopathological studies**

Kidney and lung sections that were 3 \(\mu m\) thick were cut and stained with hematoxylin and eosin for routine morphological study. Renal tissues fixed by 3% glutaraldehyde were fixed in 1% osmium tetroxide for 2 h, dehydrated by acetone, and embedded by epoxide resin. Semithin sections were then fixed optically and ultrathin sections were double electron stained with uranyl acetate-lead citrate for ultrastructural study by an H-600 IV transmission electron microscope (AMRAY, USA).

**Immunofluorescence studies**

Fresh renal tissues of rats in \(6 \times 10^8\) PFU RSV60 and normal control were snap-frozen in liquid nitrogen and cut on a cryostat. Sections (5 \(\mu m\) thick) were air-dried and fixed in cold acetone for 10 min. After washing with phosphate-buffered saline, the sections were stained with polyclonal fluorescein isothiocyanate-conjugated antibodies to IgG, IgA, IgM, and C3. The immunofluorescence findings were visualized according to the localization of deposits in the renal tissues.

**In situ hybridization**

RSV-specific probes composed of three oligonucleotides with sequences chosen from the published sequence of the G protein mRNA of RSV (sequence I: 5’-cttcctgccatgctgcgcgctgcgttggcttg-3’; sequence II: 5’-gagtttctgacctagttccagtcttct-3’; sequence III: 5’-tcctggctctctgctgtgctgtgctttcct-3’) were synthesized by Genebase Biology Company (Shanghai, China). The oligonucleotides were end-labelled with digoxigenin and hybridization was carried out by a hybridization detection kit according to the manufacturer’s instruction (DakoCytomation, Denmark) and reference on \textit{in situ} hybridization, with minor modifications. Kidney and lung sections (5 \(\mu m\) thick) were deparaffinized with xylene, rehydrated through decreasing concentrations (100–95–85–70%) of ethanol, inactivated with endogenous enzymes by 3% hydrogen peroxide at room temperature for 5–10 min, and digested with 0.1 mg/ml proteinase K at 37°C for 10 min. Prehybridization solution was then added to the
sections, which were kept at a temperature of 38–42°C for 2–4 h. Hybridization was carried out by treating with 20 µl hybridization solution containing 25–50 ng probes and keeping overnight in a wet cabinet supplemented with 20% glycerine at 38–42°C. The following day, sections were rinsed successively twice in 2 × standard sodium citrate, once in 0.5 × standard sodium citrate, and once in 0.2 × standard sodium citrate for 15 min each, treated sequentially with confining liquid, biotinylated mouse anti-digoxin, streptavidin biotin complex and biotinylated peroxidase, colored with 3,3′-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin. The slides containing RSV-infected HeLa cells served as positive controls and the sections treated without probes served as negative controls. 

Judgement of result. The positive hybridization signal locating in cytoplasm presented as dark brown. The images were collected by an image analytical system (Nikon & Spot, Japan). Five visual fields were selected under the same condition and average integral optical density of each visual field was calculated for statistics.

RSV plaque assay 

A plaque assay was used to determine RSV titer in renal and pulmonary tissue.2 Sterile renal and pulmonary tissues were homogenized in precooling DMEM containing 2% FCS in a 10% ratio (wt/vol). Homogenized samples were centrifuged at 2000 g for 10 min. Serial dilutions of the supernatants were stored at −70°C. HeLa cells in DMEM containing 10% FCS were inoculated in 12-well plates. After 2 days, the supernatant was removed and substituted with DMEM containing 2% FCS when the HeLa cell monolayer was confluent, after which serial dilutions of supernatants of renal and pulmonary homogenates were inoculated onto confluent HeLa cells and incubated at 37°C. After a 1.5-h virus adsorption, supernatants were removed and cells were washed twice in D-Hanks and overlaid with DMEM containing 2% FCS and 1% methylcellulose. After 3 days, HeLa cells were fixed with 10% neutral formalin at room temperature for 10 min and stained with 0.5% crystal violet for 10 min, and the plaques were enumerated under an inverted microscope. 

Statistical analysis 

Data are presented as means ± s.d. After the test of homogeneity of variance, the Bonferroni or Dunnnett T3 test was used for group comparison, and Spearman’s correlation test was used for correlation between two results. A P < 0.05 was considered significant. Statistical analysis was carried out with Statistical Package for the Social Sciences (SPSS) version 11.0. 

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