A Mouse Model of Chlamydia pneumoniae Strain TWAR Pneumonitis

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Chlamydia pneumoniae is a common cause of acute respiratory infections in humans. We evaluated mice as experimental animals for C. pneumoniae. Intranasal inoculation of Swiss Webster mice with C. pneumoniae induced a prolonged course of lung infection, as demonstrated by reisolation of organisms from lungs (at 42 days) and persistence of lung pathology (>60 days). The lung pathology was characterized by patchy interstitial pneumonitis with predominately polymorphonuclear leukocyte infiltration in the early and mononuclear cell infiltration in the later stages of infection. Inoculated mice developed serum immunoglobulin G antibody responses and partial resistance against rechallenge inoculation. The other mouse strains tested, Icr, BALB/cAnN, C57BL/6N, C3H/HeN, and B6C3F1, were shown to be susceptible to C. pneumoniae. The mouse model should be useful for investigating the immunopathogenesis of C. pneumoniae infections.

Chlamydia pneumoniae strain TWAR has been recognized as the third species of Chlamydia (7). So far, only one serovar or strain (TWAR) has been found. The TWAR organism has been previously established as an important cause of respiratory infections, being associated with 5 to 10% of cases of pneumonia, bronchitis, and sinusitis (5, 6). Infection with TWAR is very common. Population antibody prevalence studies have shown that more than 50% of adults worldwide have antibody (5, 6). While antibody is infrequent in children under the age of 5, incidence studies have shown antibody conversion of 6 to 9%/year in children from the ages of 5 to 14, with lower rates in older persons (1). The prevalence of antibody continues to increase throughout adulthood, and it is highest in the elderly. TWAR infections, which may be more asymptomatic in the young, often result in pneumonia in the elderly. The organism is susceptible to macrolides and tetracyclines. Treatment of symptomatic illness is often successful, but eradication of the organism is difficult. Recently, C. pneumoniae has been associated with atherosclerosis and coronary artery disease (14, 17).

No information is available on the pathology of C. pneumoniae infections, and no successful animal model has been reported.

Mice are a natural host for two pneumonia-causing chlamydial agents, the meningopneumonitis strain of Chlamydia psittaci (2, 4, 9) and the mouse pneumonitis strain of Chlamydia trachomatis (15). Intranasal inoculation of mice with the human biovars of C. trachomatis, i.e., trachoma and lymphogranuloma venereum, has been previously shown to induce pneumonitis (3, 8, 12). In classifying TWAR as belonging to a separate species, C. pneumoniae, we showed that mice were susceptible to intranasal inoculation with C. pneumoniae (11). Here we report on a mouse model of pneumonitis that should be useful for studying the pathogenesis of C. pneumoniae pneumonia.

MATERIALS AND METHODS

C. pneumoniae strain and inoculum preparation. TWAR strain AR-39, a pharyngeal isolate, was used in this study (7).

AR-39 was originally isolated and propagated in HeLa 229 cell culture (11). Infected cells were harvested with sterile glass beads and ultrasonically disrupted. The cell culture organism was partially purified by 1 cycle each of low- and high-speed centrifugation, resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen in 0.5-ml aliquots at −70°C. The inoculum preparations contained 5.3 × 106 to 6.6 × 108 inclusion-forming units (IFU) of organisms per ml.

Experimental animals. Several strains of 4- to 5-week-old male mice were used, including outbred mice (Swiss Webster and Icr), inbred mice (BALB/cAnN, C57BL/6N, and C3H/HeN), and hybrid mice (B6C3F1) (Simonsen Laboratory Company, Gilroy, Calif.).

Inoculation of mice. Mice were inoculated with the organism suspension or SPG buffer by the intranasal route. Mice were lightly anesthetized by ether inhalation to induce hyperventilation. Five drops (0.05 ml) of inoculum was delivered onto the nostrils with a 23-gauge needle attached to a syringe. Delivery of inoculum was timed to the inhalation phase of respiration. Each mouse was given 2.7 × 105 to 3.3 × 107 IFU of organisms unless otherwise specified.

Infectivity assay. Mice were killed by axillary bleeding under ether anesthesia. Blood was saved to obtain serum for serological assay. Lungs were removed in toto, including trachea and bronchi. Lungs were weighed, minced with scissors, and homogenized with a mortar and pestle to make a 10% (wt/vol) suspension in cold SPG buffer. Tissue suspensions were centrifuged at 500 × g for 10 min at 4°C to remove coarse tissue debris and were frozen at −70°C until tested. The infectious titer was assayed by titration of tissue homogenates in HL cells (13) grown on a 12-mm-diameter round coverslip in a flat-bottom, 1-dram (ca. 4-ml) shell vial. Inoculated cells were incubated at 36°C for 4 days. Infected cells were fixed in acetone (19) and stained with a Chlamydia genus-specific monoclonal antibody (CF-2) conjugated to fluorescein isothiocyanate (11). Inclusion bodies were counted under a fluorescence microscope. The infectivity titer was expressed as log10 IFU per gram of lung.

Serology. Serum antibody was detected by the microimmunofluorescence test with formalin-fixed whole elementary bodies of AR-39 as antigen (18). Both immunoglobulin G

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TABLE 1. Comparison of susceptibilities of six strains of mice to intranasal inoculation with C. pneumoniae

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Genetic background</th>
<th>Result after isolation of organisms from lungs</th>
<th>Mean titer ± SD (log IFU/g of lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss Webster</td>
<td>Outbred</td>
<td>No. of deaths/ no. tested</td>
<td>No. of positives/ no. tested</td>
</tr>
<tr>
<td>BALB/cAnN</td>
<td>Inbred</td>
<td>1/8</td>
<td>4/5</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>Inbred</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>Inbred</td>
<td>4/8</td>
<td>3/4</td>
</tr>
<tr>
<td>Icr</td>
<td>Outbred</td>
<td>0/8</td>
<td>5/6</td>
</tr>
<tr>
<td>B6C3F1</td>
<td>Hybrid</td>
<td>2/8</td>
<td>4/5</td>
</tr>
</tbody>
</table>

* Intranasal inoculation was with 2.7 × 10⁷ IFU of organisms per mouse.  
  * At day 11 postinoculation.  
  * Denominators exclude those mice that died before day 11 or that were used for histopathological study.  
  * Averages for positive mice.

(IgG) and IgM serum antibody fractions were measured with fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM (heavy-chain specific; Sigma Company, St. Louis, Mo.). Antibodies were first screened with a single dilution of sera at 1:8. Positive sera were titrated by serial twofold dilutions.

**Histopathology.** Lung tissues from infected and control mice were removed from days 2 to 6 and immediately fixed in 10% buffered formalin. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.  

**Determination of intranasal ID₅₀.** Serial 10-fold dilutions of inoculum were prepared in SPG buffer. Each dilution was inoculated intranasally into four mice. Mice were killed on day 4 postinoculation to obtain lungs for the infectivity assay. The 50% intranasal infective dose (intranasal ID₅₀) was calculated by the method of Reed and Muench (16).

**RESULTS**

**Susceptibilities of different strains of mice to C. pneumoniae.** To select the most suitable strain of mouse for our experiments, we compared the susceptibilities of the six strains of mice. On the basis of preliminary studies, each mouse was inoculated with 2.7 × 10⁷ IFU of organisms and the infectivity assay was done on day 11. As shown in Table 1, all six strains of mice were susceptible to C. pneumoniae. No significant differences were found among the six strains of mice in mean titers of organism recovery from lungs (P > 0.05 by r test). Deaths were observed with Balb/cAnN, C57BL/6N, and B6C3F1 mice. Swiss Webster mice showed no deaths, homogeneous susceptibilities, and less individual variation and yielded a high infectivity titer.

**Primary infection.** Swiss Webster mice were used to study the course of primary infection. At days 2, 4, 7, 11, 15, 21, 28, 35, 42, 49, and 60 after inoculation, six to seven mice were euthanized for reisolation of the organism (four to five mice) and for histopathology (two mice). Blood was saved to obtain serum for serology. Control mice inoculated with SPG buffer were euthanized in parallel at each time point for isolation and histopathological and serological examination.

(i) **Clinical observation.** The inoculated mice showed weakness and decreased activity for the first 2 days. They consumed less food and water than controls. Ruffled fur was noted. Mice recovered within 1 week. No deaths were observed.

(ii) **Isolation.** Reisolation of organisms from lung tissues was positive until day 42 (Table 2). The infectivity titer was highest on day 2 and then decreased gradually. The isolations were positive with all of the mice tested until day 21. After day 21, some inoculated mice were negative at each test date. Control mice were consistently negative.

(iii) **Serology.** All inoculated animals developed IgG antibody. IgG antibody in the microimmunofluorescence test was first found on day 11 (Table 2). The geometric mean titer was 1:28. The antibody titers increased rapidly, reached a peak of 1:147 at day 28, and then declined gradually until the end of the experiment at day 60 (1:16). No IgM antibody was detected at the lowest dilution tested (1:8). No antibodies were detected in control animals.

(iv) **Histopathology.** Gross observation showed a patchy distribution of areas of consolidation in both lungs in the first 2 weeks. The gross appearance of the lungs gradually returned to normal after 2 weeks. The histopathology of lung infections with TWAR was characterized by irregularly distributed interstitial pneumonitis that was most severe on days 2 and 4. These lesions showed extensive infiltration of polymorphonuclear leukocytes, with exudate in alveolar spaces and bronchial lumens (Fig. 1a). Areas of lung consolidation were noted. Infiltration was still severe on days 7 and 11, with mixed mononuclear and polymorphonuclear leukocytes (Fig. 1b). After day 15, the infiltrates were mainly mononuclear cells. Mild infiltration persisted through day 49. Minimum infiltrates with foci of mononuclear cells were still noted on day 60. One striking pathological feature was the observation of perivascular and peribronchial lymphoid cell accumulations which began on day 11 and persisted through day 60, the last day of observation (Fig. 1c). No pathological changes were observed for control mice examined at each time interval (Fig. 1d).

**Secondary infection.** Rechallenge inoculations were conducted to see whether mice recovered from primary infections had developed resistance against infection with TWAR. In order to determine the optimal dosage for rechal-

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TABLE 2. Duration of lung infections and serum antibody responses in Swiss Webster mice inoculated with C. pneumoniae AR-39

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Result of isolation of organisms from lungs</th>
<th>Serum IgG titer (GMT ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positives/no. tested</td>
<td>Mean titer ± SD (log IFU/g of lung)</td>
</tr>
<tr>
<td>2</td>
<td>4/4</td>
<td>6.61 ± 0.26</td>
</tr>
<tr>
<td>4</td>
<td>4/4</td>
<td>6.39 ± 0.19</td>
</tr>
<tr>
<td>7</td>
<td>4/4</td>
<td>6.24 ± 0.52</td>
</tr>
<tr>
<td>11</td>
<td>5/5</td>
<td>5.18 ± 0.46</td>
</tr>
<tr>
<td>15</td>
<td>4/4</td>
<td>5.25 ± 0.34</td>
</tr>
<tr>
<td>21</td>
<td>4/4</td>
<td>4.80 ± 0.37</td>
</tr>
<tr>
<td>28</td>
<td>3/4</td>
<td>4.98 ± 0.46</td>
</tr>
<tr>
<td>35</td>
<td>3/4</td>
<td>4.28 ± 1.31</td>
</tr>
<tr>
<td>42</td>
<td>2/4</td>
<td>4.09</td>
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<tr>
<td>49</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0/4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Intranasal inoculations were with 2.7 × 10⁷ IFU of organisms per mouse.  
  * One control mouse was negative in each period.  
  * Averages for positive mice.  
  * GMT, geometric mean titer; IgM antibody was not detected.  
  * 0 = <1:8.
The intranasal ID$_{50}$ was determined with Swiss Webster mice. It was equivalent to $7.0 \times 10^3$ IFU. Rechallenge inoculations with 20 ID$_{50}$ per mouse were given on day 70 or 100 after the primary inoculation. Attempted isolations from lungs at days 70 and 100 were negative with four mice examined for each time.

The results showed that mice recovered from primary infections were partially resistant to reinfections (Table 3). More than half (8 of 13) of previously infected mice resisted reinfection, while only one control mouse was not infected by the 20 ID$_{50}$ inoculum when challenged on day 70 after primary infection ($P < 0.02$ by Fisher exact two-tailed test).

**TABLE 3. Resistance against reinfection of lungs with *C. pneumoniae* AR-39 in Swiss Webster mice**

<table>
<thead>
<tr>
<th>Day after challenge</th>
<th>Prior infection$^a$</th>
<th>Day 70$^b$</th>
<th>Day 100$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3/7</td>
<td>4/5</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>5/6</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2/6</td>
<td>2/5</td>
</tr>
</tbody>
</table>

$^a$ At primary infection, one group of mice was inoculated with *C. pneumoniae* (yes) and the other group was inoculated with SPG buffer (no). On days 70 and 100 after primary inoculation, all mice were inoculated with *C. pneumoniae*.

$^b$ Day of challenge after primary inoculation.

$^c$ Mean titer, log IFU/g of lung; averages for positive mice.
Partial resistance was still noted on day 100 after the primary infection. Although the difference was not statistically significant, the sample size was small.

**DISCUSSION**

In this study, we demonstrated that six strains of commonly used laboratory mice were susceptible to intranasal inoculations with *C. pneumoniae*. Swiss Webster mice were chosen as suitable for studies of *C. pneumoniae* lung infections, since they yielded high lung infectivity titers, low individual variations, and no deaths and since they are relatively inexpensive. Infections in Swiss Webster mice were shown to be prolonged. Organisms were recovered from lungs until day 42 after inoculation. Inflammatory reactions were shown to persist until at least day 60. This differs from our results with pneumonitis induced in Swiss Webster mice by the human biovars of *C. trachomatis* (3, 12). With *C. trachomatis*, isolation became negative on day 14 and lung pathology returned to normal by 10 to 14 days. The difference may reflect the pathogenic nature of the organism, with *C. pneumoniae* being a respiratory pathogen and *C. trachomatis* being an ocularogenous pathogen.

*C. pneumoniae* lung infections induced a good serum IgG response in mice, and the appearance of IgG antibody correlated with the decrease in the numbers of organisms recoverable from the lungs. Whether the presence of antibody provides any protective immunity remains to be studied. The pattern of IgG appearance in mice was similar to that for the infection with the human biovars of *C. trachomatis* (3, 8, 12), in which IgG was first detected at 10 to 14 days. In this study, we failed to show serum IgM response. IgM response to *C. trachomatis* lung infection was demonstrated by Harrison et al., but only at a titer of 1:4 (8). The lowest dilution we tested was 1:8.

Although in Swiss Webster mice TWAR organisms could be recovered from infected lungs for a relatively prolonged time, the infection was not fatal and the lung histopathology returned to normal with minor residual changes. Nor was it possible by serial passage of infected lung homogenates to develop a more pathogenic inoculum. Infectivity titers declined with passage levels, and there were no reinfections after the fourth passage (data not shown).

The histopathology of *C. pneumoniae* lung infections in mice was characteristic of interstitial pneumonitis. The inflammatory infiltrates were predominantly polymorphonuclear cells in the early stage and mononuclear cells in the later stage of infection. This situation is similar to that with *C. trachomatis* pneumonitis in mice. However, one unique finding for *C. pneumoniae* infections is perivascular and peribronchial lymphoid cell accumulations. These were observed as early as day 11, and they persisted throughout the course of observation (day 60). With *C. trachomatis* infections, these lymphoid cell accumulations were associated only with reinfection (10). Because human pathology of *C. pneumoniae* infections has not been described, it is not known whether the same lung pathological changes occur in human *C. pneumoniae* infections.

The inbred and hybrid mice tested were susceptible to *C. pneumoniae* infections, but we did not follow the course of disease. Use of inbred and hybrid mice may provide an additional tool for studying the immune mechanisms of *C. pneumoniae* infections.

We have described a mouse model of *C. pneumoniae* pneumonitis. This model offers a nonfatal, prolonged course of pneumonitis with similarities to the human disease, which should be useful for studying pathology, immunity, and treatment of *C. pneumoniae* infections. Mice that had recovered from *C. pneumoniae* infections developed partial resistance to reinfection. This finding suggests that the mouse model will be useful for studies of prevention of infection.

**ACKNOWLEDGMENT**

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**REFERENCES**