Elevated Cytokine and Chemokine Levels and Prolonged Pulmonary Airflow Resistance in a Murine *Mycoplasma pneumoniae* Pneumonia Model: a Microbiologic, Histologic, Immunologic, and Respiratory Plethysmographic Profile

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Because *Mycoplasma pneumoniae* is hypothesized to play an important role in reactive airway disease/asthma, a comprehensive murine model of *M. pneumoniae* lower respiratory infection was established. BALB/c mice were intranasally inoculated once with *M. pneumoniae* and sacrificed at 0 to 42 days postinoculation. All mice became infected and developed histologic evidence of acute pulmonary inflammation, which cleared by 28 days postinoculation. By contrast, *M. pneumoniae* persisted in the respiratory tract for the entire 42 days studied. Because *M. pneumoniae* infection and its possible role in reactive airway disease/asthma is a known significant cause of acute respiratory illness in humans, including pharyngitis, tracheobronchitis, and community acquired pneumonia. More recently it has been associated with reactive airway disease and asthma. This association with asthma is particularly intriguing. In some studies, *M. pneumoniae* has been isolated from the respiratory tract of up to 20 to 25% of asthmatics experiencing acute exacerbations (9, 25; S. Esposito, F. Blasi, C. Arosio, et al., Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., p. 700, 1999). Increased bronchoconstriction with acute infection and impaired pulmonary function, especially of small airways, for up to 3 years after initial infection has also been described (5, 14, 17, 23, 30; Esposito et al., 39th ICAAC). Recent investigations have suggested that timely and effective treatment of acute *M. pneumoniae* respiratory infection can improve the course of reactive airway disease beyond the acute episode of wheezing and can prevent the development of delerious changes in pulmonary function tests (Esposito et al., 39th ICAAC; D. Gendrel, E. Marc, F. Moulin, et al., Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., p. 661, 1999). Pulmonary structural abnormalities suggestive of small airway obstruction have been detected in children 1 to 2 years after *M. pneumoniae* pneumonia by high-resolution computed tomography with significantly increased frequency compared with controls, even though the children were treated with macrolides for 14 days. In these children, a greater antimycoplasma antibody titer was a significant risk factor for the development of abnormal pulmonary sequelae suggesting that the host immune response may play a pathogenic role (13).

While the clinical significance of *M. pneumoniae* infection is becoming evident, the pathogenetic mechanisms of disease and host response are not well defined. In fact, most related in vitro and in vivo investigations have been conducted with *Mycoplasma pulmonis*, a murine pathogen, and other mycoplasma species (7, 21, 26, 28). Previous studies of murine cytokine expression with *M. pneumoniae* pneumonia have been informative but have not quantified cytokines and chemokines over the entire course of active infection nor correlated these findings with the microbiologic and histologic stage of disease. Our laboratory previously described the microbiologic and histologic findings of experimental murine *M. pneumoniae* pneumonia up to 15 days postinoculation, at which time there was still evidence of acute infection (32). In the present study, we extend this model to 42 days, well past the resolution of pulmonary inflammation. Additionally, we describe the dynamics of the pulmonary tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin-4 (IL-4), IL-6, KC (functional IL-8), IL-10, MIP-1α, and MCP-1/JE host response over the course of infection. These cytokines and chemokines were chosen because of their proven significance in pulmonary antimicrobial host defense (16, 19) and their broad range of cytokine and chemokine class representation. Indices of respiratory physiology during infection, as assessed by whole-body unrestrained plethysmography, are also presented.
mography provides a functional assessment of illness in animal models of respiratory disease.

This study was undertaken to establish a comprehensive animal model of *M. pneumoniae* pulmonary infection. It is anticipated that this model will provide insight and a baseline for future studies designed to elucidate the pathogenesis of *M. pneumoniae* infection and its role in reactive airway disease.

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**MATERIALS AND METHODS**

**Organism and growth conditions.** *M. pneumoniae* (ATCC 29342) was reconstituted in SP4 broth and subcultured after 24 to 48 h in a flask containing 20 ml of SP4 media at 37°C. When the broth turned an orange hue (approximately 72 h), the supernatant was decanted, and 2 ml of fresh SP4 broth was added to the flask. A cell scraper was used to harvest the adherent mycoplasmas from the bottom of the flask. This achieved an *M. pneumoniae* concentration in the range of $10^6$ to $10^7$ CFU/ml. Aliquots were stored at $-80°C$. All SP4 media contained nystatin (50 U/ml) and ampicillin (1.0 mg/ml) to inhibit growth of contaminants.

**Animals and inoculation.** Methoxyflurane, an inhaled anesthetic, was used for inoculum sedation. Two-month-old mycoplasma- and murine virus-free female BALB/c mice were intranasally inoculated once (day 0) with 1.25 to 3.75 $\times 10^7$ CFU of *M. pneumoniae* in 50 μl of SP4 broth. Control mice were inoculated with sterile SP4 broth. Mice were obtained from commercial vendors (Charles River and Harlan), who confirmed their mycoplasma- and murine virus-free status. Mice were housed in filter-top cages and allowed to acclimate to their new environment for 1 week. Animal guidelines were followed in accordance with the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center at Dallas.

**Sample collection.** Mice were anesthetized with an intraperitoneal injection of 75 mg of ketamine per kg of body weight and 5 mg/kg of acepromazine before cardiac puncture. Blood was centrifuged at 3,500 $\times g$ for 10 min, and the plasma was stored at $-80°C$. Bronchoalveolar lavage (BAL) specimens were obtained by infusing 0.5 ml of SP4 broth through a 25-gauge needle into the lungs, via the trachea, followed by aspiration of this fluid into a syringe. Whole-lung specimens, including the trachea and both lungs, were collected and fixed with a 10% buffered formalin solution for histologic evaluation. Samples were obtained at 2 and 16 h and at 1, 2, 4, 7, 14, 21, 28, 35, and 42 days postinoculation. At each time, different infected mice were utilized for BAL (five to eight mice) and fixed-lung (five to eight mice) specimens. Six uninfected controls were utilized at each time point (three for BAL and three for histologic evaluation).

**Culture quantification.** Twenty-five μl of neat and serial 10-fold dilutions in SP4 broth of BAL fluid (50 μl of neat was used for the initial dilution) were immediately cultured on SP4 agar plates at 37°C, while the remainder of BAL neat specimens were stored at $-80°C$. Quantification was performed by counting colonies on plated specimens and expressed as log$_{10}$ CFU/ml. If plated dilutions were negative for growth but the corresponding 10$^{-2}$ broth dilution was positive, then the specimen was assigned a value of 20 CFU/ml, the lower limit of detection.

**FIG. 1.** Percentage of mice infected with *M. pneumoniae* by culture and PCR in BAL fluid at 0 to 42 days postinoculation.

**FIG. 2.** Mean HPS for mice inoculated with live (*n* = 5 to 8) versus dead (*n* = 3 to 5) *M. pneumoniae* (Mp) and quantitative bronchoalveolar lavage (BAL) culture (Cx). Time points are at 2 and 16 h and at 1, 2, 4, 7, 14, 21, and 28 days. Values are expressed as means ± standard errors. $\star$ $p < 0.05$ between the HPS of mice inoculated with live *M. pneumoniae* and the HPS of mice inoculated with dead *M. pneumoniae*.
BAL PCR. Selected BAL samples were evaluated by PCR using open reading frame 6 primers to *M. pneumoniae* (Abbott Diagnostics LCX analyzer) (22).

Histopathology. Histopathologic score (HPS) was determined by a pathologist who was unaware of the infection status of the animals from which specimens were taken. HPS was based on grading of peribronchiolar and bronchial infiltrates, bronchiolar and bronchial luminal exudates, perivascular infiltrate, and parenchymal pneumonia. This HPS system assigned values from 0 to 26 (the greater the score the greater the inflammatory changes in the lung) (2).

UV radiation killing of mycoplasma. An aliquot of inoculum material was exposed to UV radiation (UV Crosslinker, Fisher Biotech) for 16 h to obtain dead *M. pneumoniae* for intranasal inoculation of mice. A sample of this aliquot was cultured to confirm that it was nonviable. Mice, 6 to 10 per time point, were inoculated with dead *M. pneumoniae* and sampled in the same manner as mice with live *M. pneumoniae*.

BAL cytokines. BAL specimens up to 28 days postinoculation were assessed for concentrations of TNF-α, IFN-γ, IL-4, IL-6, KC (functional IL-8), IL-10,
MIP-1α, and MCP-1/JE by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn.).

Plethysmography. Whole-body, unrestrained plethysmography (Buxco, Troy, N.Y.) was utilized to monitor the respiratory dynamics of mice in a quantitative manner. Mice were allowed to acclimate in the unrestrained chamber, and then recordings were taken for 5 min. The experimental and control groups (n = 3 to 9 per group) of mice were monitored serially and in parallel for 28 days. Enhanced pause (Penh) is a dimensionless value that represents a function of the ratio of peak expiratory flow to peak inspiratory flow and of the timing of expiration. Penh correlates with pulmonary airflow resistance or obstruction. Penh as measured by plethysmography has been previously validated in animal models of airway hyperresponsiveness (10, 11, 24, 31).

Serology. The presence of IgM and IgG antibodies to *M. pneumoniae* in the sera of infected mice was determined by ELISA. A sample was considered positive if its optical density (OD) reading was ≥2 standard deviations above the mean OD of the control serum samples (1).

Statistics. A t test was used to compare the different groups of animals at the same time point, as most of the data were normally distributed. In the few instances where the data was not normally distributed, the Mann-Whitney rank sum test was used for comparison. The Spearman rank order test was used for correlations, as all the data taken together were not normally distributed. As *M. pneumoniae* culture, cytokine and chemokine, IgM, and IgG data were all sampled from the same mouse, raw data were used for these correlations. HPS and Penh data were each from separate mice, so median values at each time point were used for correlations involving these indices. A comparison was considered statistically significant if the P value was <0.05.

RESULTS

These results represent a compilation of data from two to four separate experiments, depending on the specific parameter.

Clinical results. The fur of the mice developed a ruffled appearance 1 to 2 days postinoculation with live (n = 5 to 8) versus dead (n = 3 to 5) *M. pneumoniae* (Mp). Controls were inoculated with SP4 broth alone (n = 3). Time points are at 2 and 16 h and at 1, 2, 4, 7, 14, 21, and 28 days. Values are expressed as means ± standard errors. *P < 0.05 between mice given live *M. pneumoniae* and control mice. **P < 0.05 between mice given dead *M. pneumoniae* and control mice. ***P < 0.05 between mice given live *M. pneumoniae* and mice given dead *M. pneumoniae*.

**FIG. 4.** Cytokines and chemokines in BAL specimens by ELISA postinoculation with live (n = 5 to 8) versus dead (n = 3 to 5) *M. pneumoniae* (Mp). Controls were inoculated with SP4 broth alone (n = 3). Time points are at 2 and 16 h and at 1, 2, 4, 7, 14, 21, and 28 days. Values are expressed as means ± standard errors. 

- *P < 0.05 between mice given live *M. pneumoniae* and control mice. 
- **P < 0.05 between mice given dead *M. pneumoniae* and control mice.
- ***P < 0.05 between mice given live *M. pneumoniae* and mice given dead *M. pneumoniae*. 

**BAL culture.** BAL cultures were positive in 100% of the mice up to 14 days postinoculation and in 75% of animals at 42 days (Fig. 1). The mean titers of positive BAL cultures ranged from 6.7 to 5.4 log_{10} CFU/ml during the first 7 days of infection and then from 3.5 to 1.6 log_{10} CFU/ml at day 28 (Fig. 2). At 35 and 42 days, the mean titers were 1.8 ± 0.2 and 1.8 ± 0.3 log_{10} CFU/ml ± standard error, respectively. All control mice had...
negative BAL cultures, as did all the mice inoculated with dead M. pneumoniae.

**BAL PCR.** M. pneumoniae PCR was performed on culture-negative BAL specimens (BAL specimens from mice inoculated with dead M. pneumoniae were not included). This increased the percentage of mice positive for M. pneumoniae detection to 75 to 100% at all time points, as shown in Fig. 1. Additionally, PCR was positive in 10 of 10 culture-positive specimens and was negative in specimens from uninfected control mice.

**Histopathology.** Inflammation in the lungs after inoculation with live M. pneumoniae was most severe during the initial 7 days postinoculation, with a mean HPS of 7 for each group of animals during this interval (Fig. 2). Thereafter, inflammation declined and the lungs appeared normal at 28, 35, and 42 days postinfection. Figure 3 demonstrates the appearance of a control mouse lung compared with a lung demonstrating peribronchial and parenchymal inflammation at the height of disease during the first week postinoculation with live M. pneumoniae. All control mice had an HPS of 0 or 1.

Mice inoculated with live M. pneumoniae exhibited a significantly \( (P < 0.05) \) greater HPS compared with the lungs of mice given dead M. pneumoniae. Mice given dead M. pneumoniae completely lacked the bronchiolar and bronchial luminal exudates that were evident in animals receiving live organisms. In mice inoculated with dead M. pneumoniae, histologic inflammation peaked at days 1 to 2 and had completely subsided by day 7 postinoculation (Fig. 2).

**BAL cytokines.** BAL concentrations of TNF-α, IFN-γ, IL-6, KC (functional IL-8), MIP-1α, and MCP-1/JE were significantly greater \( (P < 0.05) \) in the mice infected with live M. pneumoniae compared with controls, in the mice inoculated with dead M. pneumoniae compared with controls, and with the exception of IFN-γ \( (P = 0.064) \), in the groups inoculated with live compared with those inoculated with dead M. pneumoniae. IL-4 and IL-10 concentrations were not statistically different between these three groups (Fig. 4).

**Plethysmography.** Penh peaked at day 2 in the mice infected with live M. pneumoniae and was significantly \( (P < 0.05) \) elevated compared with the control group through day 28, the last day of observation (Fig. 5). Penh peaked at day 1 for the mice inoculated with dead M. pneumoniae and was significantly \( (P < 0.05) \) elevated from controls only at day 3 (Fig. 5, inset).

**Serology.** M. pneumoniae IgM was present in serum in 44% of the infected mice (Fig. 6A). The IgG titers increased from
the time of detection to day 42, at which time all the mice tested demonstrated positive titers (Fig. 6B). Correlations. *M. pneumoniae* quantitative culture demonstrated a positive correlation with Penh ($r = 0.89$, $P < 0.001$, $n = 9$). HPS also demonstrated a positive correlation with Penh ($r = 0.92$, $P < 0.001$, $n = 9$). *M. pneumoniae* IgG demonstrated a negative correlation with both quantitative culture ($r = -0.68$, $P < 0.001$, $n = 40$) and Penh ($r = -0.81$, $P = 0.01$, $n = 8$). Numerous significant correlations were also found among the cytokine and chemokine, HPS, Penh, and *M. pneumoniae* culture and antibody data from the mice inoculated with live *M. pneumoniae* (Table 1).

**DISCUSSION**

These results describe a murine model of *M. pneumoniae* pulmonary infection that can be consistently achieved with a single intranasal inoculation containing a high concentration of *M. pneumoniae*. Acute pulmonary infection occurred in all mice after a single inoculation, in contrast to previous work in our laboratory that utilized three consecutive daily inoculations. Titers of *M. pneumoniae* in BAL decreased from an initial high of 10$^6$ CFU/ml to approximately 10$^1$ to 10$^2$ CFU/ml during the first 28 days of infection and remained at this low titer to the last day of observation (day 42). Acute pulmonary

![FIG. 5. Penh at 0 to 28 days postinoculation with live *M. pneumoniae* (Mp). Inset shows Penh postinoculation with dead *M. pneumoniae*. Controls were inoculated with SP4 broth alone. Values are expressed as means ± standard errors. *P* < 0.05 between experimental and control mice ($n = 3$ to 9 per group).](http://iai.asm.org/)

![FIG. 6. (A) *M. pneumoniae* IgM values at days 0 to 42 postinoculation with live *M. pneumoniae*. Time points are at 2 and 16 h and at 1, 2, 4, 7, 14, 21, 28, 35, and 42 days. Each point represents one mouse; there are five mice per time point. O.D., optical density. (B) *M. pneumoniae* IgG values at days 0 to 42 postinoculation with live *M. pneumoniae*. Time points are at 2 and 16 h and at 1, 2, 4, 7, 14, 21, 28, 35, and 42 days. Each point represents one mouse; there are five mice per time point. O.D., optical density.](http://iai.asm.org/)
inflammation was uniformly present for 21 days postinoculation, with the peak inflammatory changes occurring at 2 to 4 days after infection. In this model, M. pneumoniae infection induced an acute inflammatory phase lasting approximately 3 weeks, followed by a state in which low titers of the organism persisted in the respiratory tract in the absence of pulmonary inflammation by histologic examination.

In humans, M. pneumoniae is reported to persist in the respiratory tract for up to several months after recovery from illness (4). Even after therapy with effective antibiotics, M. pneumoniae has been shown to persist by culture for as long as 2 months (8, 27). To our knowledge, the duration for which M. pneumoniae can be detected in the human respiratory tract after acute pneumonia as determined by PCR has not been investigated. It is known that chronic carriage may occur among people with humoral immunodeficiency (29). In a recent study that detected M. pneumoniae in the lower airways of chronic, stable asthmatics by PCR, all M. pneumoniae cultures were negative (15). It will be important to investigate the chronicity of M. pneumoniae in our model, especially since chronic infection has been postulated to lead to pathologic as well as functional changes in the respiratory tract.

We demonstrated that inoculation with dead M. pneumoniae generated in the lungs of mice a histologic inflammatory response that was significantly greater than that in controls. However, this inflammatory response was less intense and of shorter duration than that induced by viable organisms. Additionally, while dead organisms were able to induce mild peribronchial and bronchial infiltrates, perivascular infiltrates, and pneumonia, they did not induce the bronchior and bronchial infiltrates, perivascular infiltrates, which may have altered the organism’s antigens. The elucidation of these mechanisms is important in understanding the immunopathology of M. pneumoniae infection.

The mice developed a specific immunologic response to M. pneumoniae. Although almost one-half of the mice developed positive IgM titers, there was no clear pattern of IgM antibody during the 6 weeks. It should be noted that this IgM murine antibody assay is not as specific as the IgG assay and is prone to nonspecific color development. In contrast, the specific IgG response was delayed, with all animals demonstrating IgG antibody by the end of the experiment. The pattern of the M. pneumoniae serology after this point will be investigated in future studies. The importance of the specific antibody response is suggested by the significant negative correlations between IgG and M. pneumoniae culture titer and between IgG and Penh.

**TABLE 1. Correlations of M. pneumoniae culture, HPS, Penh, and IgM and IgG titers with cytokine and chemokine concentrations in BAL samples from mice inoculated with live M. pneumoniae**

<table>
<thead>
<tr>
<th>Cytokine or chemokine</th>
<th>Mp culture*</th>
<th>HPS</th>
<th>Penh</th>
<th>Serum IgM</th>
<th>Serum IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>n</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.8</td>
<td>&lt;0.001</td>
<td>43</td>
<td>0.3</td>
<td>0.41</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.12</td>
<td>0.44</td>
<td>42</td>
<td>0.43</td>
<td>0.22</td>
</tr>
<tr>
<td>IL-4</td>
<td>−0.27</td>
<td>0.06</td>
<td>45</td>
<td>0.22</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.71</td>
<td>&lt;0.001</td>
<td>39</td>
<td>0.28</td>
<td>0.43</td>
</tr>
<tr>
<td>IL-10</td>
<td>−0.01</td>
<td>0.94</td>
<td>42</td>
<td>0.28</td>
<td>0.43</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.77</td>
<td>&lt;0.001</td>
<td>41</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.74</td>
<td>&lt;0.001</td>
<td>35</td>
<td>0.78</td>
<td>0.01</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.81</td>
<td>&lt;0.001</td>
<td>44</td>
<td>0.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Mp, M. pneumoniae.
This mouse model provides a means to investigate M. pneumoniae pulmonary infection, especially as it relates to the immunopathogenesis of reactive airway disease. The possible advantages of a mouse model over other existing M. pneumoniae animal models include the ease of working with mice, the availability of murine immunologic assays and transgenic knockout, and the vast amount of existing immunologic data on mice. The significant correlations between cytokines and chemokines and markers of disease severity (M. pneumoniae culture, HPS, and Penh) in this study give fodder for future inquiries involving immunologic manipulation. This model can also be utilized to assess the effects of novel antibiotics and immunomodulators on microbiologic, histologic, immunologic, and respiratory indices during M. pneumoniae infection. Further investigations are planned to explore chronic infection in a manner similar to that described for this study, particularly to look for the presence of an altered pulmonary immunoenvironment and physiology after M. pneumoniae pneumonia, as has been suggested by studies of reactive airway disease in children and adults.

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R.D.H. and H.S.J. contributed equally to this work.

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