Airway Inflammation and Bronchial Hyperresponsiveness after Mycoplasma pneumoniae Infection in a Murine Model

Richard J. Martin, Hong Wei Chu, Joyce M. Honour, and Ronald J. Harbeck
Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado

The interaction between chronic infection and chronic asthma is receiving increased investigation as a factor in the pathophysiology of asthma. To further understand this interaction, we used an animal model (BALB/c mice) with a Mycoplasma pneumoniae respiratory infection. Mice were studied 3, 7, 14, and 21 d after infection. Bronchial hyperresponsiveness (BHR) was assessed by methacholine challenge and was significantly heightened in the infected mice compared with saline controls at Days 3, 7, and 14. The associated inflammatory response was mainly neutrophils. The tissue inflammatory score significantly correlated to BHR (r = 0.78, P < 0.0001). Additionally, tissue interferon (IFN)-γ was significantly suppressed at Days 3 and 7 in the infected group compared with controls; and at Days 3, 7, and 14 compared with Day 21 in the infected group. There was a significant negative correlation between lung tissue messenger RNA levels of IFN-γ and at Days 3, 7, and 14 compared with Day 21 in the infected mice (8). Thus, although not a natural mouse pathogen, M. pneumoniae respiratory infection is associated with BHR in this murine model. It appears that acute mycoplasma infection suppresses it produces.

Increasingly, investigation into the relationship between chronic asthma and chronic infection has suggested that Mycoplasma pneumoniae and/or Chlamydia pneumoniae are present in a large proportion of asthmatic patients (1–5). This is an important finding because it suggests an infectious contribution to asthma pathophysiology and ultimately may lead to new therapeutic strategies. To better investigate the effects of these bacteria on airway function, animal models will need to be developed to study the pathophysiologic alterations that are induced by the bacterial infection.

Respiratory infection with M. pulmonis in a murine model is commonly used because this is a natural pathogen for mice (6). However, it is not a human pathogen. Pietsch and colleagues demonstrated that mice infected with M. pneumoniae expressed proinflammatory cytokines similar to those found in human asthma (7). Recently, Wubell and colleagues investigated the pathogenesis of acute M. pneumoniae respiratory infection in BALB/c mice (8). Thus, although not a natural mouse pathogen, M. pneumoniae respiratory murine infection can be used to investigate the pathophysiologic and inflammatory effects it produces.

The present study was designed to determine the alterations in bronchial hyperresponsiveness (BHR) and the associated inflammatory responses over a 3-wk time interval in a mouse model of M. pneumoniae respiratory infection.

Materials and Methods

Organism

M. pneumoniae (strain FH, ATCC 15531) was grown in SP-4 broth for 72 h at 37°C (9). Organisms were harvested, centrifuged at 10,000 × g for 20 min, washed with sterile saline, and resuspended in saline to yield approximately 1 × 10⁹ organisms/50 µL.

Animals

All experimental animals used in this study were covered by a protocol approved by the Institutional Animal Care and Use Committee. BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). They were quarantined for 4 wk before the experiment and bled to establish that they were virus-free, as indicated by negative antibody titers to six common murine pathogens. They were also negative for M. pulmonis. The mice were housed in autoclaved microisolation cages bedded with autoclaved pine chips (Sani Chips; J. P. Murphy Forest Products, Montville, NJ) using standard barrier techniques. The diet consisted of water and Purina 5015 Mouse Chow. After infection with M. pneumoniae, the infected mice and their saline controls were housed in a flexible film isolator (Model #M20; Isotec-Harlan Sprague Dawley, Indianapolis, IN) in the P3 facility of our vivarium.

Inoculation for Groups

Mice were inoculated with either M. pneumoniae or saline at Day 0. Before the inoculation, all the mice were intraperitoneally anesthetized with Avertin (ethanol) at 0.25 g/kg. Mice in the infected group were inoculated intranasally with 50 µl of M. pneumoniae containing 1 × 10⁶ colony-forming units. A similar 50-µl inoculation of saline was given to the mice in the control group.

Measurement of Airway Resistance

After a single inoculation of either M. pneumoniae or saline, BHR testing to increasing doses of methacholine (Mch) with resultant airway resistance measurements was performed in mice at Days 3, 7, 14, and 21. There were nine mice in each group at each time point.

The BHR test was performed in anesthetized, tracheostomized mice mechanically ventilated in a body plethysmograph using a modification of methods described by Martin and colleagues (10). Mice were initially anesthetized with 90 mg/kg intraperitoneal pentobarbital sodium (Abbott Laboratories, North Chicago, IL), and the trachea was exposed. A metal 19-gauge endotracheal catheter was inserted and was sutured in the trachea. After surgery, the mice were placed in a plethysmograph and the tracheostomy tube was attached to a four-way connector (Y-Tc 13/4, Small Parts, Miami Lakes, FL), with one port connected to a catheter measuring airway opening pressure (Pao) and two ports connected to the inspiratory and expiratory ports of a volume cycled ventilator (Model 8SN-480-7; Tokyo, Japan). The mice were ventilated at a rate of 160 breaths/min, with a tidal volume of 0.4

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Address correspondence to: Richard J. Martin, M.D., National Jewish Medical and Research Center, 1400 Jackson St., Denver, CO 80206. E-mail: martinr@njc.org

Abbreviations: bronchoalveolar lavage fluid, BALF; bronchial hyperresponsiveness, BHR; interferon, IFN; methacholine, Mch; messenger RNA, mRNA; airway opening pressure, Pao; polymerase chain reaction, PCR; pulmonary resistance, Rₚ; reverse transcription, RT.

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ml and 2 to 4 cm H₂O positive end-expiratory pressure. Transpulmonary pressure was estimated as the Pao, referenced to pressure within the plethysmograph. Pao approximates transpulmonary pressure in the mouse, inasmuch as the chest wall contributes little to the overall compliance of the respiratory system. Changes in volume were determined by pressure changes in the plethysmographic chamber referenced to pressure in a reference box using a differential pressure transducer (Validyne CD19A Carrier Demo, Validyne Engineering, Northridge, CA), electronically phased with a volume-cycled ventilator (Model 680; Harvard Apparatus, South Natick, MA). Airway resistance was measured during the baseline period before administration of Mch, then after a saline control dose and each subsequent doubling Mch dose from 1.6 to 50 mg/ml.

Bronchoalveolar Lavage
After Mch challenge, a bronchoalveolar lavage (BAL) was performed using 1 ml of saline in all mice. The BAL fluid (BALF) was analyzed for cell count and differential, mycoplasma culture, and polymerase chain reaction (PCR) for *M. pneumoniae*.

Histologic Analysis
After lavage, the lungs were excised. Part of the lung tissue was taken for mycoplasma culture and reverse transcription (RT)–PCR for interferon (IFN)-γ. The rest of the lung was immersed in 4% paraformaldehyde and fixed in the same solution overnight at 4°C. Lung tissue specimens were then embedded in paraffin and cut at 4 μm. Hematoxylin and eosin (H&E)–stained lung sections were evaluated under the light microscope using a histopathologic inflammatory scoring system as described previously in a hamster *M. pneumoniae* infection model (12). A final score per mouse on a scale of 0 to 26 (least to most severe) was obtained on the assessment of quantity and quality of peribronchial and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

Mycoplasma Culture
Minced lung tissue (approximate total size 5 × 5 × 5 mm) and 200 μl BALF were collected from mice in both infected and saline control groups. The samples were cultured at 37°C in SP-4 broth for up to 4 wk. After a week of culture in SP-4 broth, an aliquot (about 50 μl) of culture media was transferred, plated on PPLO agar plates, and incubated at 37°C for 3 more weeks.

*M. pneumoniae* PCR
After 6 wk of incubation, the culture solution was centrifuged and the resulting pellet was used for DNA extraction. The extracted DNA was analyzed by PCR using specific primer sets for either the P1 adhesion gene or the 16S ribosomal RNA (rRNA) gene of *M. pneumoniae* (1). The sizes of PCR products for P1 and 16S gene were 103 and 260 base pairs (bp), respectively. To further confirm the PCR specificity, 16S rRNA gene PCR products (five positive and five negative) were tested by Southern blot analysis using a 32P-labeled specific oligonucleotide probe.

RT-PCR
RT-PCR was performed to detect IFN-γ messenger RNA (mRNA) expression in the lung tissue from both infected and saline control mice. Total cellular RNA was isolated from the lung using a microscale RNA isolation kit (5′-3′ Prime Inc., Boulder, CO). RT was performed on 2 μg of total RNA as previously reported (13). After RT, the complementary DNA (cDNA) for IFN-γ was amplified using the mouse IFN-γ primers (Clontech, Palo Alto, CA). The cDNA for β-actin was also amplified as a control using mouse β-actin primers (Clontech). PCR was performed in a 50-μl reaction mixture containing 0.4 μM of each primer, 50 mM Tris-HCl (pH 8.3), 15 mM KCl, 0.2 mM of each dNTP, 1.5 mM MgCl₂, and 0.04 U/μl Taq DNA polymerase (GIBCO Life Science, Gaithersburg, MD). The PCR reactions were carried out on a DNA Thermal Cycler GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) for 35 cycles using the following step cycle: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Aliquots (25 μl) of the PCR products were electrophoresed in a 1.6% agarose gel, stained with ethidium bromide, and photographed. The specific PCR products for IFN-γ and β-actin are 365 and 540 bp, respectively. IFN-γ and β-actin bands were quantitated by densitometry (NIH Image Software; NIH, Bethesda, MD). IFN-γ/β-actin ratio was used to represent IFN-γ mRNA expression levels.

Statistics
The outcome variables were analyzed by using the Kruskal–Wallis test for continuous responses. For correlative analyses the Spearman rho was used (14).

![Figure 1](Image 319x69 to 543x191)

![Figure 2](Image 319x202 to 533x319)

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**Figure 1.** The percent positivity of *M. pneumoniae* by culture and PCR in BALF (A) and lung tissue (B).
Results
Detection of *M. pneumoniae*
Figure 1A shows the detection of *M. pneumoniae* by culture and PCR in the BALF from infected mice. There was 100% detection at Day 3 in culture and Days 3 and 7 by PCR. The other time points ranged from 40 to 70% detection. Detection of *M. pneumoniae* in the lung tissue had a positivity similar to that seen in the BALF (Figure 1B). All control group days were negative for *M. pneumoniae*.

BHR
Figure 2 shows that BHR on Day 3 was significantly increased at 12.5, 25, and 50 mg/ml and on Day 7 at 25 and 50 mg/ml compared with the control mice (*P* < 0.05). On Day 14, the 25 mg/ml dose showed a significant difference (*P* < 0.05) and a trend at 50 mg/ml. No differences were found at Day 21.

Inflammation
Figure 3 shows the BALF total white cell count and the cell differential at the different time points. This was mainly a neutrophilic response with significantly elevated time points at Days 3 and 7 in the infected groups compared with controls (*P* < 0.05). Correspondingly, the macrophages and lymphocytes were decreased on Day 3 (*P* < 0.05) and elevated on Days 14 and 21, respectively (*P* < 0.05).

The histology score (Figure 4) demonstrated significant increases for the infected group on Days 3, 7, and 14 (*P* < 0.05) compared with control. The lung tissue at Day 3 showed the most intense inflammatory response, characterized by peribronchiolar, bronchial, and perivascular infiltrates; parenchymal pneumoniae; and bronchial luminal exudate (Figure 5). There were large numbers of neutrophils and mononuclear cells in the inflammatory sites. After Day 3, the inflammatory response was seen mainly around the bronchioles and blood vessels, with decreasing numbers of both neutrophils and mononuclear cells. At all time points, tissue eosinophils were rarely seen. No inflammation was observed in saline control mice.

There was a highly significant correlation between the tissue inflammation score and airway resistance to Mch (Figure 6), *r* = 0.78, *P* < 0.0001.

IFN-γ mRNA Expression
The expression of IFN-γ mRNA in the lung tissue was significantly depressed in the infected groups on Days 3 and 7 (*P* < 0.03) compared with the control groups (Figure 7). In the infected group, the positivity of IFN-γ expression was significantly higher on Day 21 than on Days 3, 7, and 14 (*P* < 0.002). There was a significant negative correlation (Figure 8) between IFN-γ/β-actin and Mch airway resistance (*r* = −0.50, *P* = 0.022); whereas the suppression of IFN-γ appeared to allow BHR to increase, and its recovery to an elevated level (Figure 7) appeared to decrease BHR.

Discussion
A murine model of *M. pneumoniae* respiratory infection was developed to evaluate the alterations in BHR and air-
way inflammation produced by this microorganism. The acute effect, at 3 d, demonstrated a neutrophil response associated with increased BHR. The increase in BHR was also seen at Days 7 and 14, which corresponded to the tissue inflammatory score being elevated through Day 14. At Day 21 the inflammatory response and BHR were similar to the control population. Indeed, the lung tissue inflammatory score had a high correlation with BHR ($r = 0.78$, $P \leq 0.001$), as shown in Figure 6. Of potential importance was the relationship between the tissue expression of IFN-$\gamma$ mRNA and BHR. It appeared that *M. pneumoniae* respiratory infection suppressed IFN-$\gamma$ at Days 3 and 7 with a trend at Day 14, and as the infection waned at Day 21 there was a significant increase in IFN-$\gamma$ (Figure 7). The IFN-$\gamma$ mRNA levels were significantly correlated in a reverse fashion to lung resistance ($r = -0.50$, $P = 0.022$).

Our murine model of *M. pneumoniae* infection produced an acute lung tissue inflammatory response similar to that reported by Wubbel and coworkers (8).

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**Figure 5.** Histology of mouse lung tissue. Saline-treated mouse lung (A) at Day 3 shows no inflammatory response. *M. pneumoniae*-infected mouse lung at Day 3 (B and C) shows intensive inflammatory responses. View in C amplifies part of B and details the inflammatory infiltrates around a bronchiole (yellow arrowhead) and a blood vessel (green arrowhead), in the airway lumen (green arrow) and lung parenchyma (black arrow). Neutrophils, mononuclear cells, and alveolar macrophages are the main components of the inflammatory infiltrates. (H&E staining; original magnification: A and B, $\times 200$; C, $\times 400$).

**Figure 6.** Correlation between the tissue inflammatory score and BHR at a Mch dose of 50 mg/ml on Days 3, 7, 14, and 21 after infection.

**Figure 7.** IFN-$\gamma$ mRNA expression level in the infected group (open bars) is significantly depressed at Days 3 and 7 after infection compared with controls (filled bars). At Day 21, in the infected group, IFN-$\gamma$ is significantly greater than at Days 3, 7, and 14. *$P < 0.03$ control versus infected groups; **$P < 0.002$ for Day 21 in the infected group compared with other days.
sensitization in the absence of eosinophils and IgE increases. This finding has also been documented by others (17–19).

In summary, we found that a murine model of acute respiratory mycoplasma infection can induce BHR. The involved mechanism needs further elucidation, but may be linked to IFN-γ suppression. As IFN-γ increased, BHR decreased. With regard to human asthma, chronic reoccurring mycoplasma or chlamydia infection may modulate IFN-γ and produce a state of chronic BHR. Additionally, the interaction between chronic infection and atopy may further modulate IFN-γ and BHR in the pathophysiology of asthma. This murine model of mycoplasma respiratory infection can serve to enlighten our knowledge of this potential interaction.

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References


