**Mycoplasma pneumoniae** Pneumonia in a Mouse Model

Loretta Wubbel, Hasan S. Jafri, Kurt Olsen, Sharon Shelton, Beverly Barton Rogers, Ginger Gambill, Padma Patel, Enid Keyser, Gail Cassell,1 and George H. McCracken

To investigate the pathogenesis of acute *Mycoplasma pneumoniae* infection, BALB/c mice were anesthetized with metofane, and *M. pneumoniae* was introduced intranasally on days 0, 1, and 2. Mice were sacrificed on days 0–15. A histopathologic scoring system defined inflammatory changes in the lungs on a scale of 0–26 (least to most severe). Broth cultures were positive for all nasal passage and bronchoalveolar lavage (BAL) specimens. Histopathologic scores ranged from 0 to 21. The mean log10 (cfu/mL) were 4.1–6.4 on days 1–10 and ≥1.7 on days 13–15 for nasal passage and BAL specimens. Serum polymerase chain reaction was negative. ELISA for serum IgM and immunoblots for *M. pneumoniae* antibody were positive in 21 (62%) of 34 and 33 (97%) of 34 infected animals, respectively, at days 8–15. ELISA for IgG antibody was negative. This mouse pneumonia model can be used to study the immunologic and therapeutic responses to acute *M. pneumoniae* infection.

*Mycoplasma pneumoniae* was first identified as a human pathogen in 1944. Clinical studies evaluating colonization rates, factors predisposing to disease, therapeutic effects of antibiotic therapy, and the host’s immune responses are difficult to perform, necessitating assessment in animal models. Animal models of *M. pneumoniae* pneumonia have been established for hamsters, guinea pigs, and cotton rats [1, 2]. There are no data for *M. pneumoniae* mouse pneumonia models for nasal passage wash and bronchoalveolar lavage (BAL) cultures, polymerase chain reaction (PCR) in IgM and IgG serology, or for immunoblots for lung histopathology.

This study was undertaken to establish a mouse model for the study of the acute phase of pulmonary infection by *M. pneumoniae*. The model would provide baseline data for use in detailed evaluation of factors affecting colonization and immunopathogenicity and for rapid diagnostic techniques and treatment regimens.

**Materials and Methods**

*Organism and culture media.* *M. pneumoniae* ATCC 29342 (American Type Culture Collection, Rockville, MD) was reconstituted in SP4 broth [3] and subcultured after 24–48 h in a culture flask containing 20 mL of SP4 media. The flask was incubated at 37°C, and 72 h later the supernatant was decanted. Two milliliters of fresh SP4 broth was added; a cell scraper was used to remove *M. pneumoniae* from the sides of the flask. Aliquots were stored at −70°C.

**Animals.** Female *Mycoplasma* and murine virus–free BALB/c mice (ages 7–11 weeks and weighing 18–20 g) were used. Their virus-free status was confirmed by use of sentinel mice at Charles River Breeding Laboratories (Wilmington, MA). Mice were housed in filter-top cages and allowed to acclimate to the new environment for 1 week.

**Sedation for inoculation.** Metofane (Pitman-Moore, Mundelein, IL), a nonflammable, inhaled anesthetic, was used for sedation. To determine the effect of metofane on *M. pneumoniae*, 1 mL of *M. pneumoniae* ATCC 29342 was exposed in a closed jar to metofane vapors. A control dish of the organism was placed in room air. Twenty-five microliters was removed from each dish at 30-s intervals for 5 min and then every 5 min for a total of 15 min. Samples were placed on SP4 agar and incubated at 37°C, and colonies were counted on days 5–7.

**Inoculation.** Inoculation was done on days 0, 1, and 2 in control and infected mice. In the latter, 50 µL of broth containing 5 × 10^6 cfu/mL *M. pneumoniae* was delivered intranasally by micropipette, and each mouse was held upright for 15 s. Control animals were inoculated with sterile broth.

**Sample collection.** An intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg acepromazine provided sedation before cardiac puncture. The anterior chest wall and mandible were removed exposing the sinuses, trachea, and lungs. Individual blood, nasal passage, bronchoalveolar lavage, and lung specimens were collected from 5–10 mice on days 0, 1, 2, 4, 6, 8, 10, 13, and 15 as described below.

**Cardiac puncture.** Whole blood was obtained via cardiac puncture and centrifuged at 3500 g for 10 min. The serum was stored at −70°C.

**BAL.** A 25-gauge needle was inserted between the tracheal...
rings, and a Dieffenbach Serrefine forceps was placed on the hub of the needle. After a 1-mL tuberculin syringe was filled, 0.3 mL of SP4 culture broth was washed in and out of the bronchial tree 3–5 times, and 100 μL of the fluid was immediately processed for culture; the rest was stored at −70°C.

*Nasal passage lavage.* The proximal end of the trachea was removed revealing a small opening to the nasal passages. A 25-gauge needle was inserted into the nasal passage opening, and 0.3 mL of SP4 culture broth was flushed in and out 3–5 times. In total, 100 μL of fluid was processed for culture; the rest was stored at −70°C.

*Fixation of the lungs.* A 10% formalin solution (2 mL) was instilled into the bronchial tree, and the trachea, lungs, and heart were placed in formalin.

*Sample cultures.* We diluted 100 μL of BAL and sinus wash samples 10-fold to 10⁻² in SP4 broth. After 24 h, 25 μL of each dilution was plated on SP4 agar plates and incubated at 37°C.

*Reading and interpretation.* Broth cultures usually turned orange on days 3–6, indicating growth of *Mycoplasma* organisms. Aliquots were stored at −70°C. Random samples from positive broth cultures were evaluated by PCR in the laboratory of one of us (G.C.) using 16S primers to confirm the presence of *M. pneumoniae* by the method of Williamson et al. [4]. Agar plates were monitored weekly, and organisms were enumerated using a dissecting microscope. The broth and agar plates were considered negative if there was no growth after 6 weeks of incubation.

*Serum PCR.* Random samples of serum were evaluated by PCR in our laboratory using open-reading frame 6 primers to *M. pneumoniae* (Abbott Diagnostics, Abbott Park, IL) [5].

*Histopathology.* A histologic scoring system adapted from a hamster *M. pneumoniae* pneumonia model was applied to our model [6]. Lung specimens were evaluated for the percentage of involved sites with peribronchiolar and bronchial infiltrates, quality of infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

*Immunologic studies.* IgM and IgG antibodies to *M. pneumoniae* were determined in mice as described previously for detection of *Mycoplasma pulmonis* antibodies in mice, except that 1% bovine serum albumin–PBS was used to block the plates and dilute the samples [7–9]. The *M. pneumoniae* antigen was prepared as described for ELISA for detection of antibodies in humans [10, 11]. A sample was considered weakly positive if its optical density (OD) reading was ≥2 SD above the mean value of the control serum samples and strongly positive if the OD reading was ≥3 SD above the mean value of normal serum.

*Immunoblotting of sera* (diluted 1:100) for detection of *M. pneumoniae* antibodies was done using methods previously described [12, 13]. Hyperimmune rabbit sera and human sera previously shown to be IgM- and IgG-positive by ELISA were used for comparison as was serum from normal control mice. A sample was considered to be immunoblot-positive if it was reactive with proteins of 29.6, 52.2, 87.5, or 131.4 kDa. These represent proteins that are specific for *M. pneumoniae*.

### Results

*Clinical findings.* The animals showed no respiratory signs of pneumonia; however, their hair appeared ragged on days 3 and 4 and returned to healthy condition by day 8.

*M. pneumoniae* and metofane exposure. Exposure to metofane vapors did not result in significant reduction of numbers of *M. pneumoniae* recovered by culture.

*Serum PCR.* Twenty serum samples selected from mice with the highest colony counts in BAL on days 4, 6, 8, 10, 13, and 15 were evaluated by PCR. A positive result was defined as >500 absorbance units. The mean absorbance for all samples was 43 (range, 34–71).

*Bronchoalveolar lavage.* Broth cultures were positive in all infected mouse specimens collected from days 0 to 15 of the experiments and negative in all control mice. All 8 randomly selected BAL specimens were PCR-positive for *M. pneumoniae*. The mean *Mycoplasma* titers (log₁₀ cfu/mL) on days 0, 1, 2, 4, 6, 8, 10, 13, and 15 were 6.8, 6.0, 6.3, 6.4, 5.6, 5.4, 4.1, 1.7, and 1.7, respectively (figure 1).

*Nasal passage wash.* Broth cultures of nasal passage washes were positive in all infected mouse specimens and negative in all control animals. Five randomly selected wash specimens were PCR-positive for *M. pneumoniae*. The mean titers on days 0, 1, 2, 4, 6, 8, 10, 13, and 15 were 5.5, 4.2, 4.6, 5.2, 5.5, 5.1, 5.3, 3.6, 3.1, and 2.4 log₁₀ cfu/mL, respectively.

*Histopathology.* This experiment was performed in duplicate, and 76 animals were combined from days 0 to 15. On day 0, all lung specimens had normal histology. There were 68 mice in total for days 1–15. An inflammatory response was demonstrated on day 1 after inoculation and persisted throughout the experiment. Peribronchiolar, bronchial, and perivascular infiltrates and luminal occlusion and parenchymal pneumonia were present. The greatest inflammatory response was observed on days 1–4 (histopathology scores, 5–21). The heaviest confluent parenchymal infiltrates occurred on day 4 (figure 1). The
mean histopathology score on days 0, 1, 2, 4, 6, 8, 13, and 15 was 0, 11, 12, 14, 9, 8, 4, and 6, respectively.

**Immunoglobulin and immunoblot studies.** For all healthy control and infected mice, the mean OD IgG readings were <0.002 and <0.005, respectively. The mean OD IgM reading for control mice was 0.065 (0.015 SD). In contrast, the mean IgM value for infected mice increased progressively from an OD of 0.069 on day 0 to 0.131 on day 15 of the experiments. Twenty-one (62%) of 34 and 17 of 34 IgM antibody values were 2 and 3 SD, respectively, higher than IgM values for control mice at or after day 8. Immunoblots for *M. pneumoniae* antibody were positive in 33 (97%) of 34 animals, including immunoblots for 13 mice that were negative by ELISA at or after day 8. All immunoblots were negative at <6 days of infection.

**Control animals.** Control mice were sacrificed on days 0 (1 mouse), 1 (1), 2 (2), 5 (2), 11 (2), and 15 (2). All controls had negative BAL and nasal passage cultures, immunoblot, and IgG and IgM antibodies for *M. pneumoniae* and histopathologic scores of 0 or 1.

**Discussion**

This mouse *M. pneumoniae* pneumonia model provides new opportunities for analyzing the molecular pathophysiology and course of acute infection by this organism in vivo. The model uses a human *M. pneumoniae* pathogen to produce pneumonia in the mouse as demonstrated by histologic and immunologic techniques. Three consecutive days of intranasal inoculation induced disease in infected mice but not in control mice. We made multiple inoculations because we found that a single inoculum with ATCC 15331 in mice produced colonization only without histopathologic changes (data not shown).

Sedation for inoculation of organisms is critical. Metofane is an inhaled anesthetic agent approved by the Food and Drug Administration for use in animals. It requires minimal precautions and produces rapid sedation, and because respirations remain at normal levels during intranasal inoculation, organisms are evenly distributed throughout the bronchial tree. Exposure of *M. pneumoniae* to vaporized metofane did not inhibit growth of the organism.

We tested mouse serum samples by PCR because others have used the procedure as a marker for disease [14]. In that study, mycoplasmal bacteremia in children with pneumonia was rarely detected by PCR but was frequently positive with *Mycoplasma* central nervous system disease in the absence of pneumonia. The data from our model suggest that serum PCR is not a reliable indicator of pneumonia in the mouse.

This animal model uses the same mouse to compare all samples. The histologic scoring system used was chosen because it provides a reproducible system to identify small differences in the pulmonary inflammatory response. In this model, control animals did not have an inflammatory response, but infected animals had extensive bronchopneumonia.

The final step in this animal model was to demonstrate that disease caused by *Mycoplasma* organisms was associated with an immunologic response in the early phase of infection. Although there are no standardized assays in the mouse using ELISA and immunoblot for detection of *M. pneumoniae* antibodies, we formulated baseline immunologic data based on ELISA values from humans and from the *M. pulmonis* mouse pneumonia model [7, 10]. Both ELISA and immunoblot were performed, since discordance in results can occur if some epitopes are destroyed during the denaturing steps, and some nonprotein antigens can be missed. All positive IgM ELISA samples were ≥2 SD above the control mouse values. Twenty-five (75%) of 33 positive immunoblots obtained at or after day 8 correlated with an ELISA IgM ≥1 SD above the mean control mouse values. Although the disease was mild and the clinical course short, future detailed studies evaluating the later phases of infection will help define the immunologic response to *M. pneumoniae* in the mouse pneumonia model.

**Acknowledgment**

We thank Wayne Lai for his invaluable recommendations and guidance involving sample collection techniques.

**References**