Effects of Respiratory Mycoplasma pneumoniae Infection on Allergen-Induced Bronchial Hyperresponsiveness and Lung Inflammation in Mice

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Received 4 October 2002/Returned for modification 14 November 2002/Accepted 26 November 2002

Airway mycoplasma infection may be associated with asthma pathophysiology. However, the direct effects of mycoplasma infection on asthma remain unknown. Using a murine allergic-asthma model, we evaluated the effects of different timing of airway Mycoplasma pneumoniae infection on bronchial hyperresponsiveness (BHR), lung inflammation, and the protein levels of Th1 (gamma interferon [IFN-γ]) and Th2 (interleukin 4 [IL-4]) cytokines in bronchoalveolar lavage fluid. When mycoplasma infection occurred 3 days before allergen (ovalbumin) sensitization and challenge, the infection reduced the BHR and inflammatory-cell influx into the lung. This was accompanied by a significant induction of Th1 responses (increased IFN-γ and decreased IL-4 production). Conversely, when mycoplasma infection occurred 2 days after allergen sensitization and challenge, the infection initially caused a temporary reduction of BHR and then increased BHR, lung inflammation, and IL-4 levels. Our data suggest that mycoplasma infection could modulate both physiological and immunological responses in the murine asthma model. Our animal models may also provide a new means to understand the role of infection in asthma pathogenesis and give evidence for the asthma hygiene hypothesis.

Asthma is a common but complex syndrome that affects people worldwide. It has been suggested that one of the key reasons for this uncontrolled health problem is that in developed countries children in their early life are much less exposed to infectious agents (1, 22). This idea led to the initial hygiene hypothesis that asthma and allergic diseases could be reduced by infection in early childhood (22). Asthma represents a shift from a Th1 immunological response to a Th2 process, and infection directs the immune system toward the reverse, i.e., Th1 response. Thus, the present hygiene hypothesis in asthma states, “The immune system in newborn infants is skewed toward Th2 cells and needs timely and appropriate environmental stimuli (e.g., infection) to create a balanced immune response” (1). These Th1 stimuli include Mycobacterium tuberculosis, measles virus, hepatitis A virus, and increased exposure to various infections through contacts with other people, thereby balancing the Th1-Th2 response. Some of the recent studies of both human subjects and animal models have further supported this hypothesis (9, 12, 13, 21, 24, 25).

Respiratory infection with atypical bacteria (mycoplasma and chlamydia) has drawn increasing attention in clinical asthma research (2, 10, 15, 18, 26). A previous investigation showed that >50% of chronic stable asthma patients had evidence of airway infection with Mycoplasma pneumoniae, and these individuals had the greatest number of mast cells in their airway tissue (18). In a double-blind, placebo-controlled trial, antibiotic treatment significantly improved pulmonary function only in those asthmatics with M. pneumoniae infection (16). These observations suggest a close link between chronic asthma and chronic airway M. pneumoniae infection. In a further investigation of the role of airway M. pneumoniae infection, it was reported that bronchial hyperresponsiveness (BHR) and airway inflammation increased in a mouse model with M. pneumoniae infection (17). These human and animal studies have left several important questions to answer. First, according to the hygiene hypothesis, could early M. pneumoniae infection prior to allergen exposure suppress the asthma response? Second, could M. pneumoniae infection after allergen exposure worsen airway inflammation and function? In the present study, we intended to answer these questions in

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FIG. 1. (A) Time line for M. pneumoniae infection followed by OVA sensitizations and challenges. (B) Time line for OVA sensitizations and challenges followed by M. pneumoniae infection.
a murine allergic-asthma model by infecting the animals with *M. pneumoniae* before and after allergen sensitization. Our results show that *M. pneumoniae* infection prior to allergen sensitization suppresses airway inflammation and BHR. In contrast, *M. pneumoniae* infection after allergen exposure increases airway inflammation and BHR.

**MATERIALS AND METHODS**

Organism. *M. pneumoniae* (strain FH; ATCC 15531) was grown in SP-4 broth for 72 h at 37°C, harvested, spun at 10,000 × g for 20 min, washed with sterile saline, and resuspended in saline to yield ~10^8 CFU/50 μl (17). 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 (4 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine). They were quarantined for 4 weeks before the experiment and bled to establish that they were virus and *Mycoplasma pulmonis* free. 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, Ind.) in the P3 facility of our vivarium. (A P3 containment unit enables working with biosafety level 3 hazards.)

*M. pneumoniae* inoculations. Mice were inoculated with either *M. pneumoniae* or saline. Before the inoculation, all the mice were intraperitoneally anesthetized with Avertin (ethanol) at 0.25 g/kg of body weight. Mice in the infected group were inoculated intranasally with a single 50-μl dose of *M. pneumoniae* at ~10^9 CFU. A 50-μl inoculation of saline was similarly given to the mice in the control groups.

Ovalbumin sensitization and challenge procedure. Mice were intraperitoneally sensitized twice in a span of 14 days (day 1 and day 14) by injection of 20 μg of ovalbumin (OVA) emulsified in 2.25 mg of aluminum hydroxide (AlmImuject; Pierce, Rockford, Ill.). Fourteen days after the last OVA sensitization, the mice were placed in a Plexiglas chamber and challenged with 1% aerosolized OVA for 30 min using the ultrasonic nebulizer (De Vilbiss). The OVA challenges were given once daily for 3 days (23). Animal models. (i) *Mycoplasma-OVA sensitization-OVA challenge model*. As illustrated in Fig. 1A, mice were either infected with *M. pneumoniae* or treated with saline 3 days prior to OVA sensitization and challenge. On day 35 postinfection or after saline treatment (2 days after the last OVA challenge), the mice were tested for BHR and airway inflammation.

(ii) OVA sensitization-OVA challenge-mycoplasma model. The OVA sensitization-OVA challenge-mycoplasma model was designed to elucidate the effects of mycoplasma infection on mice with allergic experimental asthma induced by OVA sensitization and challenge. BHR and airway inflammation were evaluated on days 3, 7, 14, and 21 after infection or days 5, 9, 16, and 23 after the last OVA challenge (Fig. 1B).

Measurement of airway resistance. As previously reported (17), airway resistance (in centimeters of H₂O per milliliter per second) was measured to indicate BHR at the baseline, with a saline control, and after each subsequent doubling methacholine dose from 1.6 to 100 mg/ml. To ensure experimental reproducibility, the airway resistance measurement in each experimental group was repeated at least twice with a variation of ~10%. The log of the dose of methacholine required for a 200% increase of airway resistance over the baseline (log PC<sub>200</sub> in milligrams per milliliter) was calculated for each mouse to represent the...
degree of BHR. Lower log PC200 values represent greater airway resistance or BHR.

**Analyses of airway inflammation.** (i) BAL. After methacholine challenge, a bronchoalveolar lavage (BAL) was performed using 1 ml of saline in all mice. Three hundred microliters of the BAL sample was analyzed for the total and differential cell counts, mycoplasma culture, and PCR for *M. pneumoniae*. The remaining BAL sample was centrifuged, and the supernatant was collected and stored at −80°C for cytokine measurement.

(ii) Histological analysis. After BAL, the lungs were removed and excised. Part of the left lung tissue (approximate total size, 5 by 5 by 5 mm) was taken for mycoplasma culture. The rest of the lung was fixed and the resulting pellet was used for DNA extraction. The extracted DNA was amplified in a double-blinded fashion under the light microscope using a histopathologic scoring system as described previously in a hamster *M. pneumoniae* infection model (3) and mouse models of *M. pneumoniae* infection (11, 17). A final score per mouse (both infected and uninfected) on a scale of 0 to 26 (least to most severe) was obtained based on an assessment of the quantity and quality of peribronchiolar and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

(iii) IFN-γ and IL-4 protein measurement in BAL fluid. Levels of gamma interferon (IFN-γ) and interleukin 4 (IL-4) proteins in BAL fluid were measured by using mouse IFN-γ and IL-4 sandwich enzyme-linked immunosorbent assay kits from R&D Diagnostics (Minneapolis, Minn.). The assay detection limits are ≥9.4 pg/ml for IFN-γ and ≥7.8 pg/ml for IL-4.

Mycoplasma culture and *M. pneumoniae* PCR. Mice were killed by cervical dislocation on day 5 after *M. pneumoniae* challenge. Part of the left lung tissue was removed and excised. The remaining lung was fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 4 μm. H&E-stained lung sections were evaluated in a double-blinded fashion under the light microscope using a histopathologic scoring system as described previously in a hamster *M. pneumoniae* infection model (3) and mouse models of *M. pneumoniae* infection (11, 17). A final score per mouse (both infected and uninfected) on a scale of 0 to 26 (least to most severe) was obtained based on an assessment of the quantity and quality of peribronchiolar and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

Statistics. If the data were normally distributed, they were presented as means ± standard errors of the mean, and the data were compared between groups by the t test. When the data were not normally distributed, the data were expressed as medians with interquartile (25-to-75%) ranges, and the comparisons between the groups were performed by using the Wilcoxon rank sum test. Pairwise comparisons for contrasting lung histology scores between groups on different days were made via analysis of variance (20). A two-tailed P value of <0.05 was considered statistically significant.

**RESULTS**

*M. pneumoniae* infection prior to allergen sensitizations and challenges. Log PC200 was used to represent BHR to methacholine challenge. Lower log PC200 values represent greater BHR. On day 35 after *M. pneumoniae* infection and day 2 post-allergen challenge, log PC200 was significantly higher (P = 0.002) in infected plus OVA-treated mice compared with saline-plus-OVA-treated mice (Fig. 2). Therefore, BHR was markedly reduced in infected mice.

The IFN-γ protein level in BAL fluid was higher in infected plus OVA-treated mice than in saline-plus-OVA-treated mice (Fig. 3A). On the other hand, the IL-4 protein level tended to be lower in infected plus OVA-treated mice (Fig. 3B). As a result, the Th1/Th2 (IFN-γ/IL-4) ratio was significantly higher in infected plus OVA-treated mice than in saline-plus-OVA-treated mice (Fig. 3C).

The total white cell count in BAL was significantly lower (P = 0.03) in infected plus OVA-treated mice than in saline-plus-OVA-treated mice. Although the percentages of macrophages, neutrophils, lymphocytes, and eosinophils were similar in the two groups, the absolute cell counts for these individual types of inflammatory cells tended to be lower in infected mice (Table 1). The lung tissue inflammation scores, however, were

### TABLE 1. BAL cell profile in *M. pneumoniae*-OVA model

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Differential (%)</th>
<th>Total (10^3/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline + OVA</td>
<td><em>M. pneumoniae</em> + OVA</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td><strong>Total white</strong></td>
<td>252.5 (198.8–351.3)</td>
<td>165.0 (100.0–185.0)</td>
</tr>
<tr>
<td><strong>Macrophage</strong></td>
<td>68.0 (67.9–93.5)</td>
<td>84.8 (73.8–93.5)</td>
</tr>
<tr>
<td><strong>Neutrophil</strong></td>
<td>0.7 (0–2.0)</td>
<td>0.1 (0–0.7)</td>
</tr>
<tr>
<td><strong>Lymphocyte</strong></td>
<td>3.0 (0.5–6.0)</td>
<td>1.6 (0.3–3.8)</td>
</tr>
<tr>
<td><strong>Eosinophil</strong></td>
<td>11.5 (2.8–19.5)</td>
<td>9.0 (4.2–24.4)</td>
</tr>
</tbody>
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*a* Data expressed as median (interquartile range).

*b* P < 0.05 compared with Saline + OVA group as calculated by Wilcoxon rank sum test.

FIG. 4. Measurement of BHR to aerosolized-methacholine challenge in mice which were sensitized and challenged with an allergen (OVA) followed by *M. pneumoniae* (MP) infection or saline treatment as shown in Fig. 1B. BHR measurement was performed on days 5, 9, 16, and 23 post-allergen challenge (days 3, 7, 14, and 21 postinfection) in Allergen + saline and Allergen + MP mice. Lower values of log PC200 represent greater BHR. The data are expressed as medians. n = 8, 6, 14, and 9 for allergen-challenged plus saline-treated mice on days 5, 9, 16, and 23 post-allergen challenge, respectively. *, P < 0.05 (allergen-challenged plus saline-treated versus allergen-challenged plus MP-infected mice).
not different in the two groups (infected, 9.1 ± 0.9; saline treated, 10.1 ± 0.9; \( P > 0.05 \)).

On day 35 postinfection, 1 of 8 (12.5%) infected plus OVA-treated mice was positive for \( M. \) pneumoniae in BAL and lung tissue by PCR, but not by culture. None of the saline-plus-OVA-treated mice was positive for \( M. \) pneumoniae either by culture or by PCR.

**\( M. \) pneumoniae infection after allergen sensitizations and challenges.** The time course of BHR as represented by log PC200 is shown in Fig. 4. On day 5 post-allergen challenge (day 3 postinfection), there was a significant increase in log PC200 (decrease of BHR) in infected mice compared with saline-treated mice. However, from days 9 to 16 post-allergen challenge (days 7 to 14 postinfection), BHR was significantly increased in infected mice. On day 23 post-allergen challenge (day 21 postinfection), the BHRs in the two groups were similar.

IFN-\( \gamma \) protein levels in BAL fluid showed a trend similar to that of log PC200 (a trend opposite that of BHR) (Fig. 5A). Compared with saline-treated mice, infected mice tended to have an increase in IFN-\( \gamma \) on day 5 post-allergen challenge and then a decrease in IFN-\( \gamma \) from days 9 to 23. Conversely, IL-4 protein levels in the BAL fluid of infected mice decreased slightly on day 5, followed by a significant increase in this Th2 cytokine on day 9 (Fig. 5B). On day 16, the IL-4 level in infected mice was about twofold higher than that in saline-treated mice. On day 23, IL-4 levels in both groups returned to the day 5 level (Fig. 5B). The IFN-\( \gamma \)/IL-4 ratio also had a trend similar to that of log PC200 (a trend opposite that of BHR) (Fig. 5C). This ratio was higher in infected mice on day 5 but lower from days 9 to 16. Interestingly, when BHR started to increase in infected mice on day 9, the IFN-\( \gamma \)/IL-4 ratio also changed significantly to a Th2 phenotype. On day 23, the IFN-\( \gamma \)/IL-4 ratios in the two groups of mice were similar.

In infected mice, the percentage of neutrophils on day 5 and the BAL total cell count on day 9 (Table 2) were marginally increased, since considerable interanimal variability existed. From days 16 to 23, the BAL inflammatory-cell profiles in the two groups were similar. However, lung tissue inflammation was consistently higher in infected mice throughout the experiment (Fig. 6A). The lung inflammation during the first week was characterized by infiltrate with large numbers of neutrophils and eosinophils (Fig. 6B and C). The increased lung inflammation from days 16 to 23 was mainly due to the accumulation of mononuclear cells and a few eosinophils around the bronchioles and blood vessels, but without alveolar-wall infiltrate (Fig. 6D and E).

In infected mice, \( M. \) pneumoniae positivities in BAL and/or lung tissue by PCR were 75, 62.5, 44.4, and 33.3% on days 5, 9, 16, and 23 post-allergen challenge (days 3, 7, 14, and 21 postinfection, respectively).
on days 5, 9, 16, and 23 post-allergen challenge, respectively. None of the saline-treated mice were positive for \textit{M. pneumoniae} either by PCR or by culture.

**DISCUSSION**

For the first time, we have shown that \textit{M. pneumoniae} infection can modify the allergic responses in a murine model of asthma. Our data support the hygiene hypothesis for asthma in that \textit{M. pneumoniae} infection prior to allergen sensitization and challenge protected the animals from the BHR and airway inflammation seen in the control mice. On the other hand, if infection happened after allergen sensitization and challenge, BHR and airway inflammation increased.

Respiratory tract bacterial infections are common in childhood (6, 14), but the role of bacterial infection in asthma is still controversial. Some studies showed no association of early childhood bacterial infection and subsequent wheezing and/or asthma (4, 7). Other studies, however, demonstrated that endotoxin exposure during the first year of life could either minimize the risk of allergy sensitization or increase the risk of wheezing in children with a family history of allergy or asthma (9, 19). \textit{M. pneumoniae} is an atypical pathogenic bacterium in the human respiratory system. It is one of the common causes of community-acquired pneumonia (5). Retrospective studies indicate that children with a previous history of \textit{M. pneumoniae} infection may have a higher incidence of asthma or wheezing (6). These studies need to be carefully interpreted, because the exact timing of the infection and the confirmation of the pathogens might not be accurate. In the present study, we explored the effects of \textit{M. pneumoniae} infection on the pathogenesis of a murine allergic-asthma model. BHR was selected as a key parameter for the allergic response. In addition, we evaluated Th1 (IFN-\(\gamma\)) and Th2 (IL-4) cytokine responses and airway inflammation to elucidate the potential mechanisms behind the changes found in BHR.

In the infected-allergen-sensitized–allergen-challenged model, mycoplasma infection decreased BHR compared with saline treatment prior to allergen challenge. This reduced BHR in infected mice was accompanied by an increased Th1 and a decreased Th2 response. The numbers of BAL inflammatory cells, including eosinophils, were also decreased in the mice that were infected following allergen challenge. Our data support the hygiene hypothesis in that mycoplasma infection prior to allergen contact could induce a Th1 response, which is believed to be protective against the allergic response (8, 22). Therefore, \textit{M. pneumoniae} may be a new member of a family of infectious pathogens that could be beneficial for the prevention of asthma and other allergic diseases if the infection precedes the allergen(s).

Although respiratory infections could be linked to the acute exacerbation of asthma, the timing of the infection may be crucial to the outcome of infection in asthmatics or subjects with a family history of allergy or asthma. After we tested the hygiene hypothesis, we evaluated the effects of mycoplasma infection after allergen sensitizations and challenges were performed. We found that in our allergen-sensitized–allergen-
**Figure A**

- **Lung histology score** vs. **Day**
  - **Allergen + MP**
  - **Allergen + saline**

- **p = 0.002**

**Figure B**
- Microscopic image of lung tissue with various histological features.

**Figure C**
- Another microscopic image highlighting different structures.

**Figure D**
- Further microscopic image with noted anatomical details.

**Figure E**
- Additional microscopic image showcasing specific cellular structures.
challenged–infected model, there was a significant decrease in BHR on day 5 after allergen challenge (day 3 postinfection). From days 9 to 16 after allergen challenge, however, BHR was significantly increased. The Th1-Th2 balance in infected mice showed a trend opposite that of BHR, and this may in part explain the biphasic BHR response after infection. Generally, the pattern of Th1 and Th2 cytokine response in allergen-challenged and saline-treated mice was opposite that in allergen-challenged and infected mice. From days 5 to 16 after allergen challenge, IFN-γ increased and IL-4 decreased in saline-treated mice. This suggests that the timing after the last allergen challenge and/or infection could substantially affect the Th1 and Th2 cytokine levels in the lung, which should be considered in the interpretation of the data. In addition to the effects of Th1-Th2 balance, increased lung tissue inflammation in infected mice may also be involved in higher levels of BHR at the late time points of the experiment. These results indicate that mycoplasma infection in allergic asthma may actually be protective in the acute phase of the infection, which would challenge the traditional concept that infection always exacerbates asthma. Respiratory tract infection with M. pneumoniae in clinical patients is usually insidious. Bronchial hyperresponsiveness observed in the early phase of the infection would suggest that acute mycoplasma infection in asthma patients could not easily be identified. However, the long-term effects of the infection might be deleterious.

It is important to point out that the pattern of BHR in the allergen-sensitized–allergen-challenged–infected model is opposite that in the single M. pneumoniae infection model without any allergen exposure, where BHR initially increased and then decreased (17). In addition, the positivity of M. pneumoniae in the allergen-sensitized–allergen-challenged–infected model was also lower than in the single-infection model, which showed 100% M. pneumoniae positivity during the first week of infection. The differences in BHR, inflammatory response, and M. pneumoniae positivity between these two studies indicate a complex interaction between the allergic inflammatory response and mycoplasma infection. The allergen-induced inflammatory milieu may also affect the activity of M. pneumoniae in the lung.

Finally, it is worth mentioning that our present study evaluated only the interactions between a single mycoplasma infection and relatively short-term allergen challenges. Future studies need to determine the long-term effects of repeated and prolonged infections on BHR, airway inflammation, and, perhaps more importantly, airway remodeling, which are important features of asthma. This will further our understanding of asthma pathophysiology and treatment.

ACKNOWLEDGMENTS

We thank Misoo C. Ellison for her assistance in statistical analysis. This research was supported by the American Lung Association Asthma Research Center.

REFERENCES


Editor: J. N. Weiser