The Impact of Steroids Given with Macrolide Therapy on Experimental *Mycoplasma pneumoniae* Respiratory Infection

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**Background.** Systemic steroids have been advocated in addition to antimicrobial therapy for severe *Mycoplasma pneumoniae* pneumonia. We evaluated the efficacy of clarithromycin, dexamethasone, and combination therapy for *M. pneumoniae* respiratory infection.

**Methods.** Mice infected with *M. pneumoniae* were treated with clarithromycin, dexamethasone, combined clarithromycin/dexamethasone, or placebo daily; mice were evaluated at baseline and after 1, 3, and 6 days of therapy. Outcome variables included *M. pneumoniae* culture, lung histopathologic score (HPS), and bronchoalveolar lavage cytokine, chemokine, and growth factor concentrations.

**Results.** Clarithromycin monotherapy resulted in the greatest reductions in *M. pneumoniae* concentrations. After 3 days of treatment, combination therapy significantly reduced lung HPS compared with placebo, clarithromycin, and dexamethasone alone, whereas, after 6 days of therapy, clarithromycin alone and combination therapy significantly reduced lung HPS compared with placebo. Concentrations of interleukin (IL)–12 p40, RANTES, macrophage chemoattractant protein–1, and cytokine-induced neutrophil chemoattractant were significantly lower in mice treated with clarithromycin alone and/or combination therapy compared with dexamethasone alone and/or placebo; combination therapy resulted in a significantly greater reduction than clarithromycin alone for IL-12 p40 and RANTES.

**Conclusions.** Although monotherapy with clarithromycin had the greatest effect on decreasing levels of cytokines and chemokines as well as pulmonary histologic inflammation.

*Mycoplasma pneumoniae* is a common etiology of pediatric and adult community-acquired pneumonia, causing 10%–40% of cases [1–4]. Treating *M. pneumoniae* pneumonia with appropriate antibiotics, such as macrolides, has been found to significantly improve the course of disease in both animal models and human investigations [5–12]. Observational data from both children and adults have indicated that the addition of systemic steroids to antimicrobial therapy may improve the outcome of severe *M. pneumoniae* pneumonia. As a result of this clinical observation, systemic steroids have been advocated in addition to antibiotic therapy for severe *M. pneumoniae* pneumonia [13–16]. Steroid therapy has been found to be of possible benefit for the treatment of inflammation related to some infectious diseases, such as certain types of bacterial meningitis [17, 18]. Alternatively, steroid therapy has been shown to be of no value for other infectious diseases, such as bronchiolitis, and may be harmful [19, 20].

In addition, evidence of acute *M. pneumoniae* infection is found in up to 20% of acute asthma exacerbations in adolescents and adults [21–25]. For more severe asthma exacerbations, systemic steroids are given while antibiotics are not routinely administered, because the microbiologic etiology of asthma exacerbations is not...
frequently determined in routine practice. Some evidence does suggest that appropriate antimicrobial therapy may be of value in the treatment of M. pneumoniae–associated exacerbations of wheezing; however, more definitive data are needed [21, 26, 27]. Additionally, evidence suggests that macrolides may have anti-inflammatory properties independent of their antimicrobial effect [5].

The specific and comparative effects of treatment with macrolides, systemic steroids, or their combination on M. pneumoniae respiratory tract infection has not been fully investigated. The effect of systemic steroids on infection-induced airway inflammation and airway function is incompletely understood, particularly as it relates to infectious asthma. In the present study, we investigated the effect of clarithromycin, systemic dexamethasone, and combination clarithromycin/dexamethasone therapy on M. pneumoniae–induced airway inflammation in a murine model. In particular, we evaluated pulmonary histopathologic inflammation; bronchoalveolar lavage (BAL) cytokine, chemokine, and growth factor concentrations; markers of airway function; and M. pneumoniae quantification during the course of these therapies.

METHODS

Organism and growth conditions. M. pneumoniae (ATCC 29342) was reconstituted in SP4 broth and subcultured after 24–48 h in a flask containing 20 mL of SP4 medium at 37°C. When the broth turned an orange hue (after ~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 1 × 10^8 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 potential contaminants.

Animals and inoculation. Mice were obtained from commercial vendors (Jackson Laboratories), which confirmed their mycoplasma- and murine virus–free status. The Animal Resource Center at the University of Texas Southwestern Medical Center performed quarterly health surveillance on sentinel mice housed in the mouse storage room. Antibodies against mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reo-3 virus, mouse encephalitis virus (GD-7), mouse rotavirus (epizootic diarrhea of infant mice), minute virus of mice, and Mycoplasma pulmonis were analyzed in sentinel mice. Sentinel mice were also screened for pinworm and mites. The sentinel mice tested negative for these pathogens. Mice were housed in filter-top cages and allowed to acclimate to their new environment for 1 week. Isoflurane, an inhaled anesthetic, was used for inoculum sedation. Female BALB/c mice (9–12 weeks old) were

Figure 1. Quantitative Mycoplasma pneumoniae cultures of bronchoalveolar lavage (BAL) samples from mice inoculated with M. pneumoniae and treated with clarithromycin alone, dexamethasone alone, combined therapy, or placebo for 6 days (treatment began 1 day after inoculation). Bars represent results from 7–10 mice per treatment group at each time point from repeated experiments. Values shown are means ± SDs (error bars). Mp, M. pneumoniae. *P < .05 for the difference between the 2 specified treatment groups (1-way analysis of variance followed by pairwise multiple comparisons).
intranasally inoculated once with $1 \times 10^7$ cfu of M. pneumoniae in 50 μL of SP4 broth. All mice were housed in the same animal room and received identical daily care. Animal guidelines were followed in accordance with the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

**Treatment regimen.** Treatment was initiated 1 day after M. pneumoniae inoculation. Clarithromycin (25 mg/kg) was administered subcutaneously once daily [5]. Dexamethasone (0.5 mg/kg) was administered intraperitoneally once daily [28–30]. For the combined therapy, mice received clarithromycin (25 mg/kg) subcutaneously and dexamethasone (0.5 mg/kg) intraperitoneally once daily. Clarithromycin and dexamethasone were reconstituted in sterile 5% dextrose water. Placebo groups received sterile 5% dextrose water administered subcutaneously once daily.

**Experimental design and sample collection.** Mice were evaluated after 1, 3, and 6 days of therapy. Samples were obtained from 7–10 mice per treatment group (4 groups: clarithromycin monotherapy, dexamethasone monotherapy, combined therapy, and placebo therapy) at each time point from repeated experiments. Mice were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg acepromazine before cardiac puncture. Blood was centrifuged at 3500 g for 10 min, and the serum was stored at −80°C. BAL specimens were obtained by instilling 500 μL of SP4 broth through a 25-gauge needle into the lungs, via the trachea, followed by aspiration of this fluid into a syringe. Lung specimens, including the trachea, were collected and fixed for histologic evaluation.

**Culture.** Twenty-five microliters of undiluted BAL sample and serial 10–fold dilutions of BAL sample in SP4 broth (50 μL of undiluted BAL sample was used for the initial dilution) were immediately cultured on SP4 agar plates at 37°C, and the remaining undiluted BAL sample was stored at −80°C. Quantification was performed by counting colonies on plated specimens, and quantities were expressed as $\log_{10}$ colony-forming units per milliliter.

**Histopathology.** The histopathologic score (HPS) was determined by a single pathologist who was unaware of the treatment status of the animals from which specimens had been taken. The HPS was based on grading of peribronchiolar/bronchial infiltrate, bronchiolar/bronchial luminal exudate, perivascular infiltrate, and parenchymal pneumonia (neutrophilic alveolar infiltrate). This HPS system assigned values from 0 to 26 (the greater the score, the greater the inflammatory changes in the lungs) [31]. In our experience, the extent of variation in HPS when the same slide is scored by the same pathologist multiple times has been found to be 0–1.

**Plethysmography.** Whole-body plethysmography (Buxco), performed without restraint or sedation, was used to monitor

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**Figure 2.** Lung histopathologic score from mice inoculated with Mycoplasma pneumoniae and treated with clarithromycin alone, dexamethasone alone, combined therapy, or placebo for 6 days (treatment began 1 day after inoculation). Bars represent results from 7–10 mice per treatment group at each time point from repeated experiments. Values shown are medians and 25th–75th percentiles (error bars). *P < .05 for difference between the 2 specified treatment groups (Kruskal-Wallis test followed by pairwise multiple comparisons).
the respiratory dynamics of mice quantitatively at baseline (airway obstruction) and after methacholine exposure (airway hyperresponsiveness). Before methacholine exposure, mice were allowed to acclimate to the chamber, and plethysmographic readings were then recorded to establish baseline values. Next, mice were exposed once to aerosolized methacholine (25 mg/mouse); after exposure, plethysmographic readings were recorded. Enhanced pause (Penh) is a dimensionless value that represents a function of the ratio of peak expiratory to peak inspiratory flow, a function of the timing of expiration. Penh correlates with pulmonary air flow resistance or obstruction. Penh, as measured by plethysmography, has been previously validated in animal models of airway hyperresponsiveness [32–36].

**BAL cytokines and chemokines.** Concentrations of cytokines and chemokines in BAL specimens were assessed using multiplex bead immunoassays (Bio-Rad Laboratories) in conjunction with the Luminex LabMAP system, following the manufacturers’ instructions. The assay limits of detection, according to Bio-Rad Laboratories, are as follows: for interleukin (IL)–1β, 0.8 pg/mL; for IL-2, 1.1 pg/mL; for IL-4, 0.5 pg/mL; for IL-5, 0.8 pg/mL; for IL-6, 1.1 pg/mL; for IL-8, 0.5 pg/mL; for IL-9, 0.7 pg/mL; for IL-10, 0.9 pg/mL; for IL-12p70, 0.5 pg/mL; for IL-13, 2.1 pg/mL; for IL-17, 0.2 pg/mL; for eotaxin, 14.6 pg/mL; for granulocyte colony-stimulating factor, 1.1 pg/mL; for granulocyte-macrophage colony-stimulating factor, 4.5 pg/mL; for interferon-γ, 19.3 pg/mL; for macrophage chemotactic protein (MCP)–1, 6.7 pg/mL; for macrophage inflammatory protein (MIP)–1α, 1.1 pg/mL; for MIP-1β, 1.1 pg/mL; for platelet-derived growth factor, 1.0 pg/mL; for RANTES, 1.2 pg/mL; for tumor necrosis factor–α, 3.0 pg/mL; and for vascular endothelial growth factor, 0.5 pg/mL. For statistical analysis, samples with readings below the limit of the standard curve of the assay were assigned a value one-half that of the lowest detectable value.

**Statistics.** One-way analysis of variance was used to compare treatment groups at each time point if the data were normally distributed. In the instances where the data were not normally distributed, the Kruskal-Wallis test was used for comparisons. If a difference was found between groups, then a pairwise multiple comparison procedure was performed. A difference was considered statistically significant if $P \leq .05$.

**RESULTS**

**Culture.** Quantitative *M. pneumoniae* BAL cultures were significantly reduced for mice treated with clarithromycin alone compared with those for mice treated with placebo after 3 days.
of therapy, whereas after 6 days of therapy *M. pneumoniae* cultures for mice treated with clarithromycin alone and mice treated with combined therapy were both significantly reduced compared with those for mice treated with placebo or dexamethasone alone (figure 1).

**Lung histopathology.** The lung HPS for mice treated with combined therapy was significantly reduced after 3 days compared with the HPS for mice treated with clarithromycin alone, and dexamethasone alone (figure 2). After 6 days of therapy, the HPS was significantly lower for clarithromycin monotherapy or combined therapy than for placebo; in addition, combined therapy significantly reduced the HPS compared with dexamethasone alone (figure 2). Figure 3 demonstrates the histopathologic appearance of representative lungs after 3 days of therapy for all treatment groups.

**Plethysmography.** For airway obstruction, as measured by baseline plethysmography before methacholine exposure, no significant differences were found among the treatment groups. Airway hyperresponsiveness, as measured after methacholine exposure, was significantly lower after 3 days of therapy for all treatment groups compared with the placebo group (figure 4).

**Cytokines, chemokines, and growth factors.** BAL concentrations of IL-12 p40, RANTES, MCP-1, and cytokine-induced neutrophil chemoattractant (KC) were significantly lower for mice treated with clarithromycin monotherapy and/or combined therapy than for mice treated with dexamethasone alone and/or placebo, as depicted in figure 5. No significant differences were found for the other 21 cytokines, chemokines, and growth factors investigated.

**DISCUSSION**

*M. pneumoniae* is generally associated with mild to moderate community-acquired pneumonia that is self-limited and/or responds well to appropriate antimicrobial therapy. However, *M. pneumoniae* pneumonia may also be severe, with accompanying acute respiratory failure that may not respond promptly to appropriate antimicrobial therapy [13, 14]. Severe pulmonary injury with *M. pneumoniae* pneumonia has been hypothesized to be due to an exuberant host immune response rather than direct microbial damage [37–39]. Immunopathogenic investigations in *M. pneumoniae* pneumonia animal models support this supposition [36, 39–42]. The use of systemic steroids to diminish the host response in severe *M. pneumoniae* pneumonia, in addition to antimicrobial therapy, is supported by observational case series in both children and adults [13–16]. Although many observational and placebo-controlled, double-blind, randomized investigations have demonstrated the beneficial role of antimicrobial therapy in *M. pneumoniae* respiratory tract infection in adults, the role of systemic steroids in the treatment of severe *M.
Pneumoniae respiratory illness is not well defined [9–11]. Furthermore, systemic steroids are often proposed for the treatment of extrapulmonary manifestations of M. pneumoniae infection, particularly central nervous system manifestations, without clear data indicating the effect that steroid therapy has on these manifestations.

In an experimental model of M. pneumoniae respiratory tract infection, we found that combination therapy consisting of clarithromycin with dexamethasone significantly reduced pulmonary histologic inflammation compared with placebo, as well as with clarithromycin alone or dexamethasone alone after 3 days of therapy. After 6 days of therapy, the combination treatment group again had the lowest mean lung HPS; however, it was not significantly lower than that for clarithromycin alone. This may suggest that combined therapy is most beneficial in the early stages of inflammation or when lung inflammation is greatest, as the HPS for placebo peaked after 3 days of therapy (4 days after M. pneumoniae inoculation).

Of note, dexamethasone alone did not significantly reduce histologic pulmonary inflammation. In contrast to our steroid monotherapy results, Chu et al. [43] found that the administration of daily inhaled fluticasone propionate for 5 days, beginning 2 days before M. pneumoniae inoculation, significantly decreased pulmonary histologic inflammation in a mouse model. Bowden et al. [28] found that, in a mouse model of M. pulmonis chronic respiratory infection, the administration of intraperitoneal dexamethasone for 2 weeks significantly reduced the thickness of tracheal mucosa, used as a marker of tissue inflammation. Differences in experimental methods between these investigations and the current investigation likely explain the differing results. Our steroid monotherapy results may be applicable to untreated acute M. pneumoniae infection.

Microbiologically, as expected, therapies that included clarithromycin significantly reduced quantitative M. pneumoniae cultures, compared with therapies without clarithromycin. Dexamethasone monotherapy did not increase or decrease M.

Figure 5. Cytokine and chemokine concentrations in bronchoalveolar lavage (BAL) specimens from mice inoculated with Mycoplasma pneumoniae and treated with clarithromycin alone, dexamethasone alone, combined therapy, or placebo for 6 days (treatment began 1 day after inoculation). Bars represent results from 7–10 mice per treatment group at each time point from repeated experiments. Values shown are medians and 25th–75th percentiles (error bars). IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; MCP, macrophage chemotactic protein. *P < .05 for the difference between the 2 specified treatment groups (Kruskal-Wallis test followed by pairwise multiple comparisons).
pulmonis mouse model. Bowden et al. [28] compared treatment with dexamethasone to treatment with the antimicrobial agent oxytetracycline in the chronic M. pulmonis mouse model. Their group found that oxytetracycline significantly reduced quantitative mycoplasma cultures in lung tissue, compared with placebo, whereas dexamethasone did not. In tracheal tissue, they found that both dexamethasone and oxytetracycline significantly reduced quantitative cultures. Chu et al. [43] found that inhaled fluticasone propionate appeared to significantly reduce lung concentrations of M. pneumoniae compared with placebo while not reducing BAL M. pneumoniae concentrations. As a whole, these results seem to indicate that antimicrobials with in vitro activity against M. pneumoniae are effective in reducing concentrations of M. pneumoniae in vivo, whereas steroid monotherapy does not increase concentrations of M. pneumoniae during active infection and may actually decrease concentrations in some instances.

In contrast to the findings for M. pneumoniae culture and pulmonary histopathology, all 3 treatment regimens investigated significantly reduced methacholine airway hyperresponsiveness compared with placebo after 3 days of treatment, with no significant differences found between the regimens. However, it must be noted that many authorities regard the measurement of the parameter Penh, as performed in this investigation, to provide a limited screening of overall lung function rather than be a rigorous evaluation of pulmonary mechanics. In addition, Penh may correlate with air flow in the whole airway, rather than solely with pulmonary air flow. Chu et al. [43] noted that inhaled fluticasone propionate initiated before M. pneumoniae infection also significantly reduced methacholine airway hyperresponsiveness. The pathogenic mechanisms involved in the reduction of airway hyperresponsiveness may be different for clarithromycin and dexamethasone therapy, because the effects on the other measured outcomes, especially cytokines and chemokines, did not parallel the airway hyperresponsiveness results. Dakhama et al. [44] previously noted distinct differences in the in vitro adherence interactions of M. pneumoniae with cell culture after treatment with either erythromycin or dexamethasone. It also appears that clarithromycin and dexamethasone therapy are not significantly additive or synergistic for decreasing airway hyperresponsiveness. To speculate about the clinical implications, these findings may indicate that macrolide therapy is as effective as steroid therapy for an asthma exacerbation due to M. pneumoniae infection in terms of airway hyperresponsiveness. Macrolides have been postulated to have host immunomodulating activity; however, past investigations in our laboratory indicated that the beneficial activity of macrolides in the treatment of M. pneumoniae respiratory tract infection is antimicrobial, as opposed to resulting from a primary host immunomodulation mechanism [5, 6].

The significant differences detected for IL-12 p40, RANTES, MCP-1, and KC lend insight into the immunopathogenesis involved in M. pneumoniae respiratory disease and its treatment. Combination therapy caused the greatest reductions in the levels of these cytokines and chemokines; however, the absolute differences in concentrations between combination therapy and clarithromycin alone were minor. Dexamethasone monotherapy significantly increased RANTES and KC concentrations compared with placebo. The significant differences noted for IL-12 p40 concentrations parallel the pulmonary histopathologic results found with the investigated regimens, in contrast to results for RANTES, MCP-1, and KC. IL-12 has been previously reported to play an important role in the immunopathogenesis of M. pneumoniae respiratory tract infection, with less lung disease present in IL-12 knockout mice and more disease present with the administration of exogenous IL-12 [36, 45, 46]. Conversely, the IL-12 p40, RANTES, MCP-1, and KC results did not parallel the airway hyperresponsiveness outcomes. This may mean that other unmeasured factors are more elemental in the pathogenesis of M. pneumoniae–related airway hyperresponsiveness or that overlapping pathways are involved in airway hyperresponsiveness and that clarithromycin and dexamethasone act through different mechanisms to achieve a similar outcome of reduced airway hyperresponsiveness. The levels of these chemokines and others have been found to be elevated and/or to correlate with disease severity in mycoplasma infection [6, 36, 44, 47–50].

In conclusion, combination therapy with clarithromycin and dexamethasone is more effective in reducing M. pneumoniae–induced pulmonary inflammation than either clarithromycin or dexamethasone alone. These data lend support to the clinical observation that the addition of systemic steroids to antimicrobials may be of value in severe M. pneumoniae pneumonia. However, before final conclusions can be determined on the role of adding steroids to antimicrobial therapy to treat M. pneumoniae pneumonia, controlled clinical investigations in humans are necessary to determine the risks and benefits to patients, because the present investigation has the inherent limitation of having been done in a murine model. Currently, antimicrobials alone remain the primary therapy for M. pneumoniae pneumonia. Importantly, in our investigation, dexamethasone monotherapy was not found to reduce pulmonary inflammation. Of the cytokines and chemokines evaluated, IL-12 appeared to be the most closely linked with pulmonary histologic inflammation. The possibility of treating M. pneumoniae–associated wheezing with clarithromycin without the addition of steroids should be further investigated.

References


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