Effect of Azithromycin plus Rifampin versus Amoxicillin Alone on Eradication and Inflammation in the Chronic Course of Chlamydia pneumoniae Pneumonitis in Mice

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The effects of treatment with azithromycin plus rifampin (A+R), amoxicillin (A), or placebo (P) on the chronic course of experimental Chlamydia pneumoniae pneumonitis in mice were assessed by culture, PCR, and immunocytochemistry as well as by degree of inflammation in lung tissue. Eradication of the pathogen was significantly more frequent and inflammation in tissue was significantly reduced after treatment with A+R compared to after treatment with A or P. Combination therapy with azithromycin plus rifampin showed favorable effects in the chronic course of C. pneumoniae pneumonitis.

Chlamydia pneumoniae is frequently the cause of respiratory tract infections (17). A chronic course of lung infections has been occasionally described, and more recently, chronic C. pneumoniae infection has been associated with cardiovascular disease, prompting intervention studies with antimicrobial treatment of patients with unstable angina or recovering from myocardial infarction (1, 10, 11). However, many crucial issues regarding the treatment of C. pneumoniae infection in humans remain unclear. First, little is known about the treatment of acute C. pneumoniae infection (12). Second, established chronic infection is almost impossible to determine in humans by current noninvasive diagnostic techniques. Third, whether and to what extent treatment of acute infection may alter the course of chronic infection is unknown. Fourth, treatment of established chronic infection is problematic, since chlamydiae may survive in a persistent, noncultivable form which may not establish a chronic infection is problematic, since chlamydiae may survive in a persistent, noncultivable form which may not be amenable to antimicrobial treatment (3). Eradication of C. pneumoniae, which can possibly cause long-term inflammatory sequelae, may thus be of great interest. This may be achieved by different means, e.g., by prolonged treatment with an antimicrobial agent alone, or by a combination of agents during a shorter period of time. We have recently shown in experimental C. pneumoniae pneumonitis that short-term treatment with the in vitro synergistic combination of azithromycin plus rifampin was clearly superior to azithromycin alone or placebo with regard to isolation rates of C. pneumoniae and to detection of pathogen DNA within 3 weeks after infection (22). In clinical practice, however, empirical treatment of longer duration (about 1 week) is recommended for acute pneumonitis. The major goal of this study was to examine the effects of treatment on eradication of C. pneumoniae and on suppression of the inflammatory process in the chronic course of pneumonitis. C. pneumoniae strain AR-39 was grown in HL cells, partially purified by one cycle each of low- and high-speed centrifuga-
Lung sections cut at a nominal microtome setting of 3 μm and stained with hematoxylin-eosin were used for assessment of inflammation. Nodular mononuclear infiltrates, a striking pathologic feature of chronic *C. pneumoniae* pneumonitis observed previously (23), were identified in a semiquantitative fashion by counting all visible foci in one section from the entire embedded half lung. The numbers obtained were normalized to the area (1 cm²) of one section. The numbers of presumed diffuse infiltrates of inflammatory cells were determined by counting all nuclei in eight randomly chosen high-power fields (magnification, ×1,000) of alveolar parenchyma devoid of nodular infiltrates, and the results were averaged.

For immunocytochemical staining, 3-μm Formalin-fixed, paraaffin-embedded sections of mouse lung tissue were dewaxed, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) in a pressure cooker for 5 min. Sections were then (and following all subsequent steps) washed in Tris-buffered saline (TBS). A mix of a *Chlamydia* genus-specific antibody (clone CF-2) and a biotinylated rabbit anti-mouse immunoglobulin G (IgG) antibody (Dako, Glostrup, Denmark) was prepared and incubated for 30 min on a shaker at room temperature to allow complex formation. The concentration of CF-2 was approximately 2 μg/ml (1:500), while the secondary antibody was used at a dilution of 1:5000, corresponding to a 1.9-μg/ml concentration of specific IgGs. Antibodies were diluted in TBS containing 5% rabbit serum (Life Technologies, Paisley, Scotland) and 0.5% casein sodium salt (Sigma, St. Louis, Mo.). Slides were incubated with this complex of primary and secondary antibody for 2 h at room temperature. Controls were either incubated with a mixture in which clone CF-2 was replaced with the same concentration of an irrelevant mouse monoclonal antibody against *Aspergillus niger* glucose oxidase (clone DAK-GO5; Dako) or with the secondary antibody alone. Slides were incubated with avidin-biotin–alkaline phosphatase (1:200 in TBS). Dako) or with the secondary antibody alone. Slides were incubated with this complex of primary and secondary antibody for 2 h at room temperature. Controls were either incubated with a mixture in which clone CF-2 was replaced with the same concentration of an irrelevant mouse monoclonal antibody against *Aspergillus niger* glucose oxidase (clone DAK-GO5; Dako) or with the secondary antibody alone. Slides were incubated with avidin-biotin–alkaline phosphatase (1:200 in TBS). Finally, sections were developed in a new fuchsin-naphtol AS-BI substrate (Sigma, St. Louis, Mo.), counterstained with haematoxylin, cleared, and mounted. The proportion of animals for whom *C. pneumoniae* infection was eradicated was calculated from the course of pneumonitis among the three treatment groups was assessed by the total number of mononuclear cells and analyzed by chi-square test. The degrees of inflammation in animals for whom *C. pneumoniae* DNA was detected was considered, and the results were averaged.

Ten days after infection, *C. pneumoniae* was isolated from the lungs of all control (PBS) animals, from the lungs of some animals after amoxicillin treatment but not from the lungs of animals after treatment with azithromycin plus rifampin (Table 1). After 30 and 60 days, all lungs in all treatment groups were culture negative. *C. pneumoniae* AR-39 DNA was studied in culture-negative lung tissues. In controls and in animals treated with amoxicillin, DNA was consistently detected at all time points. The detection rate of pathogen DNA over time clearly declined after treatment with azithromycin plus rifampin (Table 1). Eradication was defined as no detection of *C. pneumoniae* in lung tissues as assessed by culture, PCR, and immunocytochemistry (ICC) (Table 1). Eradication rates increased with time from infection, being lowest after 10 days and highest after 60 days of infection. When data from all three time points were considered, *C. pneumoniae* was eradicated from only 2 of 23 lungs of control animals and 4 of 24 lungs of animals treated with amoxicillin, but from 11 of 24 lungs of animals treated with azithromycin plus rifampin (*P* = 0.007).

<table>
<thead>
<tr>
<th>Day of sacrifice and treatment group</th>
<th>No. of samples in which infection was detected/no. tested by:</th>
<th>No. of samples in which infection was eradicated/ no. tested</th>
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</thead>
<tbody>
<tr>
<td>Culture</td>
<td>PCR</td>
<td>ICC</td>
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<tr>
<td>10</td>
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<tr>
<td>PBS</td>
<td>8/8</td>
<td>ND</td>
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<tr>
<td>Azithromycin + rifampin</td>
<td>0/8</td>
<td>6/8</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0/7</td>
<td>4/7</td>
</tr>
<tr>
<td>Azithromycin + rifampin</td>
<td>0/8</td>
<td>2/8</td>
</tr>
<tr>
<td>60</td>
<td></td>
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<tr>
<td>PBS</td>
<td>ND</td>
<td>6/8</td>
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<tr>
<td>Azithromycin + rifampin</td>
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The number of mononuclear cell infiltrates decreased with time in all three treatment groups (data not shown). The number of mononuclear cells was not significantly different in the control and amoxicillin groups at all three time points. In contrast, the number mononuclear cell infiltrates was significantly reduced in animals treated with azithromycin plus rifampin compared with the number in either controls (*P* < 0.03) or amoxicillin-treated animals (*P* < 0.003) at all three time points. Nodular accumulations of mononuclear cells were observed only 30 and 60 days after infection in all treatment groups (Fig. 1). Their numbers declined from day 30 to day 60 in all but the amoxicillin-treated group. The number of lesions was not significantly different at both time points in controls and in animals treated with amoxicillin. In contrast, there were significantly lower accumulations of mononuclear cells after treatment with azithromycin plus rifampin compared to after treatment with PBS (controls) or amoxicillin.

In this study, we induced *C. pneumoniae* pneumonitis in mice and observed a chronic course for 2 months after infection. Experimental studies addressing treatment issues in *C. pneumoniae* pneumonitis have focused on the acute course of pneumonitis, either in immunosuppressed or immunocompetent mice (18, 20, 21). We have previously shown that short-term treatment of *C. pneumoniae* pneumonitis in immunocompetent mice suppressed chlamydial replication (18). However, the inflammatory process in lung parenchyma appeared not to be affected by antimicrobial treatment, and *C. pneumoniae* DNA could frequently be found in culture-negative lungs within 2 weeks after treatment. Reactivation experiments with cortisone acetate strongly suggested that *C. pneumoniae* DNA was representative of noncultivable but viable organisms (19). Most recently, we have performed in vitro susceptibility tests showing a synergistic activity of azithromycin plus rifampin, and this treatment regimen led to a higher rate of eradication of the organism from lung tissue than did treatment with azithromycin alone within 3 weeks after infection (22). Since *C. pneumoniae* can cause chronic inflammation in lung tissue, it was important to investigate treatment effects on the chronic course of pneumonitis. Our results show that combined treatment with azithromycin plus rifampin was clearly superior to no treatment or treatment with amoxicillin alone for eradication of *C. pneumoniae*. This higher eradication rate of the
pathogen was accompanied by suppression of the inflammatory process in lung tissue. Amoxicillin is not a treatment of choice for chlamydial infection. However, in vitro experiments have shown that ampicillin and amoxicillin each have a definite, but incomplete, inhibitory effect on *C. trachomatis* and *C. pneumoniae* at concentrations attainable in vivo (4, 5, 8, 15, 16). Masson et al. (20) have shown some activity of amoxicillin-clavulanate in reducing isolation rates of *C. pneumoniae* from lungs in experimental mouse pneumonitis 1 day after cessation of treatment. Based upon these observations, one cannot dismiss that amoxicillin might eventually induce more chlamydial persistence with long-term sequelae. Finally, aminopenicillins are frequently used for the empirical treatment of community-acquired pneumonia, and *C. pneumoniae* may account for 6 to 12% of these cases, leaving the long-term effects of such treatment unclear. In our study, amoxicillin was not statistically different from the placebo with regard to eradication of the organism and suppression of the inflammatory process. The mechanisms by which azithromycin combined with rifampin favored eradication early as well as late in the course of pneumonitis and suppressed chronic inflammation were not investigated. Rifampin has excellent antichlamydial activity in vitro (13, 22), but rapid emergence of chlamydial resistance in vitro after exposure to the drug alone has been described (14). A hypothetical mechanism by which the addition of rifampin may be responsible for the favorable effects in combination was provided recently by the observation that rifampin is a glucocorticoid receptor ligand with the ability to transactivate the receptor (6). Following this hypothesis, rifampin could act as an immunosuppressive agent, reactivating persistent infection and thus allowing azithromycin to act on replicating pathogens, eventually suppressing the chronic inflammatory process. Strategies to eradicate *C. pneumoniae* from tissue should be further investigated.

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REFERENCES


FIG. 1. Number of nodular mononuclear infiltrates in sections of lung tissue 30 and 60 days after infection. Each dot represents data from one animal. Three treatment regimens were studied, as follows: amoxicillin (Amoxi) (20 mg/kg s.c. b.i.d. for 7 days), azithromycin (10 mg/kg s.c. daily for 5 days) plus rifampin (20 mg/kg s.c. bid for 7 days) (Azi + Rif), and PBS (b.i.d. for 7 days). Statistical comparisons were done by Mann-Whitney test.