Effect of Azithromycin plus Rifampin versus That of Azithromycin Alone on the Eradication of *Chlamydia pneumoniae* from Lung Tissue in Experimental Pneumonitis

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Azithromycin, doxycycline, and rifampin, alone or in combination, were tested in vitro against *Chlamydia pneumoniae* AR-39. The combination of azithromycin plus rifampin showed the strongest activity and produced higher rates of eradication of *C. pneumoniae* from lung tissues than azithromycin alone in experimental mouse pneumonitis.

Chlamydia pneumoniae is a common cause of respiratory tract infections (12). Recently, *C. pneumoniae* has been associated with chronic conditions, such as cardiovascular disease (4). Therefore, studies about eradication of a pathogen which may cause long-term sequelae are of interest. It has been shown that although short-term treatment with either doxycycline or azithromycin resulted in rapid clearance of *C. pneumoniae* from lung tissues as assessed by culture in experimental pneumonitis, pathogen DNA could be frequently recovered from culture-negative lung tissues after treatment (14). Reactivation experiments using cortisone acetate strongly suggested that pathogen DNA is representative of viable organisms in a culture-negative state (15). The goals of this study were to assess in vitro the effect of various antichlamydial drugs on inclusion formation in cell cultures and to study the most active combination in our experimental animal model.

(This work was presented in part at the 98th General Meeting of the American Society for Microbiology, Atlanta, Ga., 17 to 21 May 1998 [19].)

C. pneumoniae AR-39 was grown in HL cells, partially purified by one cycle each of low- and high-speed centrifugation, resuspended in sucrose-phosphate-glutamic acid buffer, and frozen at −70°C. Before in vitro testing, cells were passaged three times in antibiotic-free culture medium. MICs were determined as previously described (11). After centrifugation, the inoculum was removed and replaced by culture media containing azithromycin dihydrate (Pfizer Research Laboratories, Groton, Conn.), doxycycline (Vibraveineuse; Pfizer, Zurich, Switzerland), and rifampin (Rimactan; Novartis, Basel, Switzerland). After 3 days, cells were stained with a *Chlamydia* genus-specific monoclonal antibody (CF-2) conjugated to fluorescein isothiocyanate. Inclusions were counted under a fluorescence microscope, and the MIC (defined as the concentration needed to achieve complete inhibition of inclusion formation in the original inoculum) was determined. Activity at various subinhibitory concentrations was determined by inoculation of four culture vials with AR-39 (3×10^4 inclusion-forming units [IFU] per vial). After centrifugation, the inoculum was removed and replaced immediately by culture media containing various subinhibitory concentrations (0.5, 0.6, 0.7 and 0.8 times the MIC) of azithromycin, doxycycline, and ri-

fampin, either alone or in combination. The number of IFU per coverslip was determined after 3 days and backcalculated to determine the concentration in 1 ml of the inoculum preparation, and the difference with regard to controls was assessed. The difference of IFU per milliliter of inoculum at the various subinhibitory concentrations compared to controls was expressed as $\log_{10}$ IFU per milliliter. Experiments using 0.7 and 0.8 times the MIC were run in duplicate.

Three- to four-week-old outbred male NMRI mice were inoculated by the intranasal route (5×10^7 IFU of strain AR-39/animal) as described previously (14). Treatment was started 2 days after inoculation. Groups of 15 to 20 animals were killed at different time points to assess viable counts of organisms in lung tissue. Lungs were removed in toto and immediately processed for culture (see below). Approximately one-third of the homogenate was frozen at −70°C for DNA detection by PCR. The data shown are pooled results of three experiments.

Two days after inoculation, animals were injected subcutaneously with either phosphate-buffered saline (PBS, twice a day [b.i.d.] for 3 days), azithromycin dihydrate (10 mg/kg of body weight once on day 1 and PBS b.i.d. for 3 days), or the combination of azithromycin (as described above) plus rifampin (20 mg/kg b.i.d. for 3 days). This dosage of azithromycin produced concentrations similar to those achieved in humans after an oral dose of 500 mg, inducing concentrations in pulmonary tissues of mice above the MIC of the drug for 48 to 72 h after injection (14). This dosage of rifampin produced concentrations in small rodents similar to those achieved in humans after an oral dose of 450 mg, inducing concentrations in lung tissue above the MIC for 6 to 15 h after injection (5).

Lungs were processed for culture as previously described (14). Cells were incubated at 37°C for 3 days, fixed with acetone, and stained with CF-2. Inclusions were counted under a fluorescence microscope.

DNA from a lung homogenate was isolated in extraction buffer (10 mM Tris [pH 8], 100 mM EDTA [pH 8], 0.5% sodium dodecyl sulfate) and treated with 20 μg of proteinase K per ml. Lysates were extracted with phenol–Tris-HCl (pH 8)–chloroform and precipitated with ethanol. PCR was done with the *C. pneumoniae*-specific HL-1 and HR-1 primer set, which results in an amplified product of 437 bp (2). Products were visualized by agarose gel electrophoresis and confirmed by Southern blot hybridization. DNA probes were labeled with the Genius labeling and detection kit (Boehringer Mannheim). Hybridization was detected by immunochemiluminescence

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TABLE 1. Antichlamydial activity of agents used at subinhibitory concentrations

Concn relative to MIC	Log$_{10}$ decrease (IFU/ml) compared to control[a]					
	Azithromycin	Rifampin	Doxycycline	Azithromycin + rifampin	Azithromycin + doxycycline	Doxycycline + rifampin
0.5×	1.3	0.2	0	2.7	2.7	1.5
0.6×	2.5	0.7	0.1	3.5	3.3	2
0.7×	3.3 ± 0.1	3.1 ± 0.6	0.6 ± 0.5	3.6 ± 1.3	3.5 ± 1.5	3.2 ± 0.4
0.8×	3.7 ± 0.8	3.7 ± 0.5	2 ± 1.3	5.5 ± 0.3	5.2 ± 0.7	3.5 ± 0.7

[a] At 0.7 and 0.8 times the MIC, the mean log$_{10}$ decrease ± the standard deviation is indicated.

(Lumi Phos 530; Boehringer Mannheim). Extraction controls and tissue controls from uninfected animals were run in parallel. All specimens were run in duplicate. To check for inhibition in PCR-negative samples, we used an internal standard constructed from a human gene fragment of unknown function carrying the sequence of the primer set used for *C. pneumoniae* DNA detection.

The proportion of culture-negative and DNA-negative lungs among the three treatment groups was analyzed by the chi-square test. Proportions between the single treatment groups were analyzed by the chi-square test with Yates correction.

The MICs of azithromycin, doxycycline, and rifampin for AR-39 were 0.1, 0.05, and 0.075 µg/ml, respectively. The results of the experiments using subinhibitory concentrations are shown in Table 1. The combinations of azithromycin with either rifampin or doxycycline showed enhanced antichlamydial activity. Based on these results, we decided to investigate the effect of azithromycin plus rifampin versus azithromycin alone in treating pneumonitis. When both first and second passages in cell culture are considered (Table 2), lung tissues were found to be culture negative only for the combination group after 10 and 14 days. Overall, during the whole course of pneumonitis, lungs were found to be sterile more frequently after treatment with the combination than after treatment with azithromycin alone or PBS. *C. pneumoniae* DNA was detected in 6 of 8 culture-negative lung tissues after treatment with PBS and in 12 of 13 and 6 of 16 tissues after treatment with azithromycin alone and azithromycin plus rifampin, respectively. We also assessed overall eradication of *C. pneumoniae*. While there was no difference between controls and azithromycin alone (numbers of culture- and DNA-negative lungs per total numbers of lungs, 2 of 43 and 1 of 54, respectively) ($P = 0.5$), azithromycin plus rifampin produced higher rates of eradication of *C. pneumoniae* in tissue (10 of 47 lungs were culture and DNA negative) ($P = 0.005$ and $P = 0.044$ compared to values for azithromycin alone and PBS, respectively).

Successful establishment of chlamydial infection was demonstrated by isolation of *C. pneumoniae* from lungs in controls. In vitro studies showed that inhibition of growth was achieved most consistently with azithromycin plus rifampin. The excellent activity of azithromycin was described previously based on results of conventional susceptibility experiments (13). However, the results of treating experimental pneumonitis with short-term administration of azithromycin alone have been disappointing (14). The activity of rifampin against chlamydiae has been known for many years. Under the stringent conditions of exposing the cells to antimicrobial agents 48 h after infection, rifampin was, along with doxycycline, the most active agent tested against *Chlamydia trachomatis* (1). Further investigation of this agent has been hampered by early reports of rapid emergence of resistant strains after multiple passages (9, 10, 18), and clinical data are scarce (3, 8, 16). These observations made it unlikely that rifampin monotherapy would be of practical value; therefore, we did not study the effect of rifampin alone in our pneumonitis model. However, tetracycline in combination with rifampin suppressed the emergence of resistant variants in tissue culture, resulting in either an indifferent or additive effect on inclusion formation (9). Our in vitro data led us to study the combination of azithromycin plus rifampin in an experimental pneumonitis model.

The combination of azithromycin plus rifampin produced significantly higher rates of eradication of *C. pneumoniae* from lung tissue than azithromycin alone early in the course of experimental pneumonitis. However, several limitations of our study must be considered. Treatment duration for complicated chlamydial infection was short. Long-term experiments with longer treatment duration, as suggested from clinical cases (6, 7, 17), are needed to confirm our observations. The presence of pathogen DNA does not establish the presence of viable organisms per se. In a previous study, however, the presence of pathogen DNA was correlated with recovery of infectious chlamydiae during treatment with immunosuppressive drugs (15). Long-term pneumonitis studies will have to correlate histopathological inflammation with the presence or absence of organisms. No immediate conclusions about the clinical use of these combinations should be drawn based upon these experimental data.

TABLE 2. Effect of treatment on isolation of *C. pneumoniae* by culture from lung tissue after two passages

No. of days after infection	No. of infected lungs/total no. of lungs per treatment group		
	PBS	Azithromycin	Azithromycin + rifampin
10	15/15	18/18	15/16
14	14/14	17/17	13/15
20	6/14	6/19	3/16
Total	35/43	41/54	31/47

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