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In Vitro Susceptibilities of *Chlamydia pneumoniae* Isolates from German Patients and Synergistic Activity of Antibiotic Combinations

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The susceptibilities of six *Chlamydia pneumoniae* type strains and of six German patient isolates to erythromycin, azithromycin, roxithromycin, clarithromycin, doxycycline, ofloxacin, and rifampin were investigated. MICs and minimal chlamydicidal concentrations were all within the ranges reported previously. Combinations of azithromycin with either ofloxacin, doxycycline, or rifampin, as well as combinations of three antibiotics (rifampin, azithromycin, and ofloxacin or doxycycline), showed synergistic activity against *C. pneumoniae*.

Chlamydia pneumoniae is an important cause of respiratory-tract infections (3, 17). Furthermore, an association of *C. pneumoniae* with atherosclerosis was detected by a serologic case control study 10 years ago (24) and was subsequently confirmed by seroepidemiological studies and by detection of the organism in atherosclerotic plaques (25). This association is of great interest because of the possibility of antibiotic treatment.

Cultivation of *C. pneumoniae* from patients' specimens is difficult. Therefore, only a limited number of *C. pneumoniae* strains is available for testing of antimicrobial susceptibility, and most data published so far were obtained either with type strains or with isolates from American patients (1, 2, 9, 13, 16, 21–23, 27).

There are only a few controlled trials of antimicrobial therapy of *C. pneumoniae* respiratory-tract infections, which is due to the fact that most infections are still diagnosed serologically. Serology does not detect all cases (3), and usually diagnosis is only retrospective. *C. pneumoniae* infections tend to be chronic, with prolonged symptoms, and relapses occur even after appropriate antibiotic therapy (5, 13, 17). Experiences with other chronic infections showed that short-term therapy with a single antibiotic did not always eradicate the infection, whereas combination therapy improved therapy outcome. Treatment of chronic *C. pneumoniae* infections might also be improved by prolonged therapy or by the combination of two or three antibiotics with synergistic effects.

C. pneumoniae FR-1 through FR-5 were isolated in our laboratory from patients with respiratory-tract infections (FR-1, FR-3, FR-4, and FR-5) and from a patient with lymphadenopathy, right heart dilatation, and pericardial effusion (FR-2). HK-J was isolated from the throat swab of a patient in Jena, Germany. The type strains examined for comparison were TW-183 and AR-39 (Washington Research Foundation [WRF], Seattle, Wash.) and ATCC VR-1310, ATCC VR-1355, ATCC VR-1356, and ATCC VR-1360 (American Type Culture Collection, Manassas, Va.). All strains were propagated to high titers by cell culture methods described previously (7). For comparison, HL cells (WRF), HEp-2 cells (Flow Laboratories, Meckenheim, Germany, United Kingdom), and BGM cells (7) were used. Erythromycin (Sigma Chemical Co., Deisenhofen, Germany), azithromycin (Pfizer, Karlsruhe, Germany), clarithromycin (Abbott Laboratories, Wiesbaden, Germany), roxithromycin (Hoechst, Bad Soden, Germany), doxycycline (Sigma), ofloxacin (Sigma), and rifampin (Sigma) were solubilized according to the manufacturers' instructions.

First, the suitability of the three different cell lines investigated in this study was evaluated by using type strain ATCC VR-1310. MICs and minimal chlamydicidal concentrations (MCCs) (given below, respectively, in micrograms per milliliter) were similar on HL and HEp-2 cells and fell within the ranges previously reported (9, 13): on HL cells, 0.125 and 0.125 for doxycycline, 0.125 and 0.125 for erythromycin, and 2.0 and 2.0 for ofloxacin; on HEp-2 cells, 0.25 and 0.5 for doxycycline, 0.25 and 0.125 for erythromycin, and 2.0 and 1.5 for ofloxacin. In contrast, the MICs and MCCs of erythromycin tended to be higher on BGM cells: 0.125 and 0.125 for doxycycline, 1.0 and 1.0 for erythromycin, and 2.0 and 2.0 for ofloxacin. Therefore, we decided to use HL cells for the following assays. For determination of the MICs and MCCs of the seven antibiotics against the 12 *C. pneumoniae* strains, a microtiter procedure was used as previously described with slight modifications (1). HL cells grown in 96-well microtiter plates (Falcon; Becton Dickinson Labware, Bedford, Mass.) were infected with 20 μl of each chlamydial strain at a concentration of 10^3 to 10^4 inclusion-forming units/ml. After centrifugation (1,960 × *g* for 60 min at 36°C), the inoculum was replaced by Eagle's minimal essential medium (supplemented with 10% fetal bovine serum and 1 μg of cycloheximide/ml) containing the antibiotics in twofold serial dilutions. Each dilution step was tested in parallel in six replicates with each chlamydial strain. After incubation at 37°C under 5% CO_2 for 72 h, infection was assessed by staining with a genus-specific, fluorescein-conjugated monoclonal antibody (Pathfinder; Kallestad Pasteur, Freiburg, Germany). The MIC was defined as the lowest concentration of antibiotic at which no inclusions were seen. The MCC was determined by removing the antibiotic-containing medium, washing twice with phosphate-buffered saline, and passaging onto new cells. The MCC was the lowest antibiotic concentration which resulted in no inclusions after this passage. All tests were run in triplicate. The MICs at which 50 and 90% of the isolates are inhibited and the MCCs at which 50 and 90% of the isolates are killed are given for seven antibiotics in Table 1.

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TABLE 1. MICs and MCCs of seven antibiotics against six *C. pneumoniae* type strains and six German isolates

Antibiotic	MIC (μg/ml)				MCC (μg/ml)			
	Range		50%	90%	Range		50%	90%
	Type strains	German isolates			Type strains	German isolates		
Doxycycline	0.125–0.25	0.125–0.25	0.125	0.25	0.0625–0.25	0.0625–0.25	0.125	0.25
Ofloxacin	2.0–4.0	2.0	2.0	2.0	1.0–2.0	1.0–2.0	2.0	2.0
Erythromycin	0.031–0.125	0.015–0.125	0.0625	0.125	0.015–0.125	0.015–0.0625	0.031	0.125
Roxithromycin	0.125–0.5	0.125–0.5	0.5	0.5	0.125–0.5	0.125–0.5	0.25	0.5
Clarithromycin	0.004–0.015	0.0075–0.015	0.0075	0.015	0.004–0.0075	0.004–0.015	0.0075	0.015
Azithromycin	0.0625–0.125	0.0625–0.125	0.0625	0.125	0.031–0.125	0.0625–0.125	0.0625	0.125
Rifampin	0.015–0.031	0.0075–0.031	0.015	0.031	0.015–0.031	0.0075–0.031	0.015	0.031

The MICs and MCCs for the German patient isolates were in the ranges obtained with the American type strains. The MICs and MCCs at which 90% of the strains were inhibited or killed, respectively (Table 1), were all within the ranges reported in the literature (1, 2, 8, 9, 16, 19–23, 27), except for those of roxithromycin (1 dilution step higher in our study) and azithromycin (the MCC was 1 dilution step lower in our study). For rifampin, comparison was not possible because of a lack of previous reports of MICs and MCCs against *C. pneumoniae*. Since rifampin was reported to be very active against *Chlamydia trachomatis* (9, 15), we decided to evaluate the MICs and MCCs of this antibiotic alone and in combinations.

For evaluation of possible synergistic effects, combinations of two or three antibiotics were tested with type strain ATCC VR-1310 on HL cells in a microtiter assay as described above. The antibiotic combinations that were tested are shown in Table 2. Combinations of two antibiotics at three subinhibitory concentrations of each (0.002 to 0.0075 μg of rifampin/ml, 0.0075 to 0.031 μg of azithromycin/ml, 0.25 to 1.0 μg of ofloxacin/ml, and 0.015 to 0.0625 μg of doxycycline/ml) were tested by a checkerboard titration method (4). Each assay included controls without antibiotics and with the MIC of each antibiotic alone. Combinations of three antibiotics were tested with three to eight serial twofold dilutions of the MIC of each antibiotic (0.0005 to 0.0075 μg of rifampin/ml, 0.0075 to 0.031 μg of azithromycin/ml, 0.0075 to 1.0 μg of ofloxacin/ml, and 0.0075 to 0.0625 μg of doxycycline/ml). The fractionary inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated as follows: FIC index = $FIC_A + FIC_B$, $FIC_A = (A)/(MIC_A)$, and $FIC_B = (B)/(MIC_B)$, where (A) is the concentration of drug A in the well that has the lowest inhibitory concentration in its dilution row and (MIC_A) is the MIC of the organism to drug A alone (4). Synergistic combinations are defined as having a FIC index of ≤0.5. The results of testing combinations of two or three antimicrobials are shown in Table 2.

To date few data have been published on the response of *C. pneumoniae* infection to antimicrobial therapy (3, 6, 13). Some data suggest that antibiotic therapy that is effective at curing *C. trachomatis* infections appears to be less appropriate with *C. pneumoniae* infections, despite comparable in vitro data for MICs and MCCs (13). One possible reason for treatment failures is resistance. However, none of the strains examined in our study showed resistance to any of the antibiotics tested. In contrast, *C. trachomatis* strains with resistance to several antibiotics have been described (14). In *C. pneumoniae* strains, primary antibiotic resistance has not yet been observed (13). However, in a study published recently, the MIC of azithromycin for three *C. pneumoniae* isolates obtained from two patients after treatment had increased fourfold (22). Another possible explanation for treatment failures might be that *C. pneumoniae* causes chronic-persistent infections, which are difficult to eradicate. Experiences in the treatment of other chronic infections, such as *Mycobacterium tuberculosis* or *Helicobacter pylori* infections, have shown that prolonged and combined therapy might be necessary to improve the results. There are two published preliminary studies on antibiotic therapy in patients with atherosclerotic diseases (10, 11), both of which suggested a benefit of short-term antibiotic therapies, but they need to be confirmed with a larger number of patients. Since short-term antibiotic therapy is usually not sufficient in cases of acute respiratory *C. pneumoniae* infection (13, 17), therapy of chronic infections can be expected to be even more difficult. A study published recently by Sinisalo et al. (26) showed that prolonged doxycycline monotherapy had no effect on *C. pneumoniae* antibody titers.

The association between *C. pneumoniae* and atherosclerosis possibly will provoke a great number of randomized and non-

TABLE 2. Combinations of two or three antibiotics

Combination			FIC index	Result
Antibiotic 1 (concn)[a]	Antibiotic 2 (concn)[a]	Antibiotic 3 (concn)[a]		
Rifampin (1/4)	Azithromycin (1/4)		0.5	Synergistic
Rifampin (1/2)	Ofloxacin (1/2)		1.0	Not synergistic
Rifampin (1/2)	Doxycycline (1/2)		1.0	Not synergistic
Azithromycin (1/4)	Ofloxacin (1/8)		0.375	Synergistic
Azithromycin (1/4)	Doxycycline (1/4)		0.5	Synergistic
Ofloxacin (1/2)	Doxycycline (1/2)		1.0	Not synergistic
Rifampin (1/8)	Azithromycin (1/8)	Ofloxacin (1/8)	0.375	Synergistic
Rifampin (1/8)	Azithromycin (1/8)	Doxycycline (1/8)	0.375	Synergistic

[a] Concentration necessary to prevent *C. pneumoniae* inclusions, expressed as a fraction of the MIC.

randomized, empiric antibiotic studies. However, there are also preliminary results indicating possible risks of such antibiotic therapy (12), and so risks and possible benefits must be carefully evaluated in controlled studies.

Ways to improve therapy outcome in an infection that must be supposed to be chronic-persistent might be either prolonged antibiotic therapy or combination therapy with two or more substances. In our study, combinations of azithromycin with either ofloxacin, doxycycline, or rifampin showed synergistic activity in vitro. Combinations of three antibiotics (rifampin, azithromycin, and ofloxacin or doxycycline) also were synergistic, requiring only 1/8 of the MIC that each antibiotic exhibited when tested alone. In vitro studies are the first step in the evaluation of possible synergistic antibiotic combinations. The next step would be to test these combinations in one of the several animal models which have been established (18, 19). If the synergistic effect of an antibiotic combination therapy is confirmed in such an animal model, this combination could then be tested for use in human infections.

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