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In Vitro Susceptibilities of *Actinobacillus actinomycetemcomitans* to a Number of Antimicrobial Combinations

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The in vitro susceptibilities of *Actinobacillus actinomycetemcomitans* to 14 antimicrobial combinations were studied by using the checkerboard titration technique. The results, expressed as the range of the fractional inhibitory concentration indices, were as follows: for metronidazole or its hydroxymetabolite combined with cefixime, 0.2 to 0.6; for moxalactam, 0.2 to 0.6; for penicillin G, 0.3 to 0.6; for tobramycin, 0.8 to 2.0; for erythromycin, 0.8 to 1.7; for ciprofloxacin, 0.2 to 0.6; for tetracycline, 0.8 to 1.2. Our observations indicated that the β-lactam antibiotics as well as ciprofloxacin act synergistically with both metronidazole and its hydroxymetabolite against *A. actinomycetemcomitans*. Synergistic interactions were independent of the individual MICs of the antibiotics tested. Erythromycin, tobramycin, and tetracycline combined with either metronidazole or its hydroxymetabolite showed additive to indifferent effects against the five strains of *A. actinomycetemcomitans*, with the fractional inhibitory concentration indices ranging from 0.8 to 2.0. *A. actinomycetemcomitans* was found to be highly susceptible to ciprofloxacin (MIC of ciprofloxacin for 90% of strains tested, 0.010 μg/ml) and cefixime (MIC of cefixime for 90% of strains tested, 0.8 μg/ml). The results indicate that in patients who are allergic to penicillin, cefixime and ciprofloxacin may be useful alternative antibiotics in combination with metronidazole for the treatment of *A. actinomycetemcomitans*-associated periodontitis.

Actinobacillus actinomycetemcomitans, a rod-shaped gram-negative coccobacillus, is a pathogen in several nonoral diseases (9, 13, 14, 21, 36) and a putative pathogen in periodontal disease (4, 7, 15, 25, 29, 30, 36). Treatment of *A. actinomycetemcomitans*-associated periodontitis by subgingival debridement has appeared ineffective with regard to eradication of *A. actinomycetemcomitans* from the periodontal area (23, 24, 31). For adequate treatment of this infection, antibiotics are required. In 1989, van Winkelhoff et al. (34) showed that mechanical treatment followed by a regimen of metronidazole and amoxicillin for 7 days is effective in eliminating *A. actinomycetemcomitans* from the infected sites. Recently, this was confirmed in a large patient group, in which more than 95% eradication of the microorganism was reported (35). An explanation for the in vivo efficacy may be the in vitro synergism against *A. actinomycetemcomitans* not only between metronidazole and amoxicillin but also between metronidazole and its hydroxymetabolite as well as between amoxicillin and the hydroxymetabolite of metronidazole (20). However, in some patients, effective treatment with the metronidazole-amoxicillin combination is contraindicated for several reasons; patients can suffer from serious adverse effects caused by amoxicillin, and patients can have allergies toward β-lactam antibiotics. An alternative antibiotic therapy for these patients is still not available.

The aim of the study described here was to investigate the interactions between several antibiotic combinations by using the checkerboard titration technique (3) and *A. actinomycetemcomitans* as the test organism. The combinations tested were metronidazole and its hydroxymetabolite combined with cefixime, moxalactam, penicillin G, tobramycin, erythromycin, ciprofloxacin, and tetracycline. As a follow-up to this study, the in vitro susceptibilities of 50 additional *A. actinomycetemcomitans* strains to cefixime, an expanded-spectrum oral cephalosporin, and ciprofloxacin, a 4-fluoroquinolone, were assessed. As a result of the present study, ciprofloxacin and two new combinations, metronidazole combined with cefixime and metronidazole combined with ciprofloxacin, were tested for their abilities to eradicate *A. actinomycetemcomitans* from patients suffering from *A. actinomycetemcomitans*-associated periodontitis.

MATERIALS AND METHODS

Bacterial strains. To investigate the interactions between the different antibiotics, a representative set of five *A. actinomycetemcomitans* strains (HG 1174, HG 1175, HG 1176, HG 1177, and HG 1178) was used. For the determination of the susceptibilities of *A. actinomycetemcomitans* to ciprofloxacin and cefixime, a panel of 50 additional *A. actinomycetemcomitans* strains was used. These strains were isolated by using selective TSBV plates (28); the strains were obtained from the periodontal lesions of 50 patients with severe periodontitis.

Determination of MICs. MICs were determined under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) by the microdilution method (2), with modifications as described previously (20), for metronidazole (Rhône Poulenc, Amstelveen, The Netherlands), the hydroxymetabolite of metronidazole (kindly supplied by Rhône Poulenc), cefixime (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), penicillin G (Sigma Chemical Co., St. Louis, Mo.), moxalactam (Sigma), ciprofloxacin (Sigma), tobramycin (Sigma), erythromycin (Sigma), and tetracycline (Sigma). The susceptibilities of the 50 *A. actinomycetemcomitans* strains to ciprofloxacin and cefixime were determined by the agar dilution method by using 10^5 CFU per spot under anaerobic and

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microaerophilic conditions (5% CO_2 in air) (16). The dilutions of the antibiotics were freshly prepared for each experiment. Stock solutions were made in anaerobically sterilized brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and were filter sterilized under anaerobic conditions. For the MIC determinations, bacterial cells were grown anaerobically overnight in brain heart infusion broth at 37°C. Then, inoculum suspensions of approximately 10^6 CFU/ml were prepared. MIC determinations were performed in 96-well, flat-bottom tissue culture clusters (Costar, Cambridge, United Kingdom). A 100-μl inoculum suspension was added to a 100-μl dilution of antibiotic, resulting in a bacterial density of approximately 5×10^5 CFU/ml. The MICs were read after incubation of the cultures in an anaerobic glove box for 36 h at 37°C. The MIC was defined as the lowest concentration at which no visible growth could be detected. For each strain, the MIC was determined twice in duplicate.

Checkerboard titrations. Checkerboard titration experiments were carried out for metronidazole in combination with cefixime, moxalactam, penicillin G, ciprofloxacin, erythromycin, tetracycline, and tobramycin. The antibiotics which were combined with metronidazole were also used for the investigation of the interactions with the hydroxymetabolite of metronidazole. The dilutions of each antibiotic used in the checkerboard titrations were prepared in the same way as described above for the MIC determinations, with final concentrations from 100 to 10% of the MIC. The checkerboard titrations were performed in 96-well, flat-bottom tissue culture clusters. Fifty microliters of each of the antibiotics used in the checkerboard titrations and a 100-μl inoculum suspension were added to each of the wells. The clusters were wrapped in plastic and moisturized paper tissue to prevent vaporization and were then incubated in an anaerobic glove box at 37°C for 36 h. Checkerboard titrations were performed twice in duplicate for each *A. actinomycetemcomitans* strain.

Determination of FICIs. To specify the degree of interaction between the antibiotics, fractional inhibitory concentrations and fractional inhibitory concentration indices (FICIs) were calculated for the wells in which no visible growth could be detected. The criteria that one uses to decide whether a combination of antibiotics acts synergistically, additively, or antagonistically are based on the accuracy of the MIC determination (20). Synergism in a two-dimensional checkerboard test is defined by FICIs of ≤ 0.5 by using twofold serial dilutions for the MIC determination (3). A more exact understanding of the interactions between two antibiotics can be obtained by determining the precise MICs (20). This can be achieved when smaller dilution steps are used. In the present study, the MICs were determined by using dilution steps of 10%. From this we deduced that synergism can be defined as FICIs of ≤ 0.7. This value is obtained mathematically by subtracting the sum of the maximal errors made in the MIC determinations (0.2) and the maximal errors made in the pipetting and diluting steps (0.1) from the theoretical FICI limit for synergism (1.0) (3).

Pilot study on the effects of ciprofloxacin, metronidazole plus cefixime, and metronidazole plus ciprofloxacin in patients with *A. actinomycetemcomitans*-associated periodontitis. Two patients with *A. actinomycetemcomitans* periodontitis were treated by mechanical debridement of the periodontal pockets. This treatment was followed by treatment with a regimen of ciprofloxacin (500 mg twice daily for 7 days). Three patients were treated by mechanical debridement followed by a regimen consisting of metronidazole plus cefixime (metronidazole, 500 mg twice daily; cefixime, 200 mg twice daily; both drugs were given for 7 days). Four patients with *A. actinomycetemcomitans*-associated periodontitis were treated by mechanical debridement followed by treatment with the combination of metronidazole plus ciprofloxacin (metronidazole, 500 mg twice daily; ciprofloxacin, 500 mg twice daily; both drugs were given for 7 days).

Samples for microbiological analysis were obtained with sterile paper points from four selected pockets which were positive for *A. actinomycetemcomitans* before treatment, pooled, and transported in reduced transport fluid to the laboratory (33). From this pooled sample, 10-fold dilutions were prepared in reduced transport fluid and were plated onto selective TSBV plates for the detection of *A. actinomycetemcomitans* (28). TSBV plates were incubated in a CO_2 incubator (5% CO_2 in air). *A. actinomycetemcomitans* was identified on the basis of its specific colony morphology on TSBV plates (star-like inner structure) and the production of catalase and other specific enzymes (27).

RESULTS

For 50 *A. actinomycetemcomitans* strains, the MICs of cefixime and ciprofloxacin for 90% of strains tested under both anaerobic and microaerophilic culture conditions were 0.8 μg/ml (range, 0.3 to 2.2 μg/ml) and 0.010 μg/ml (range, 0.001 to 0.020 μg/ml), respectively. All strains were susceptible to both antibiotics. No difference in the MIC of either antibiotic was observed under either culture condition. The MICs of metronidazole, its hydroxymetabolite, cefixime, moxalactam, penicillin G, ciprofloxacin, erythromycin, tetracycline, and tobramycin for the five *A. actinomycetemcomitans* strains used in the checkerboard titration experiments are listed in Table 1. The MIC for each strain was determined twice in duplicate, and the MICs showed little variation. The five *A. actinomycetemcomitans* strains were susceptible to cefixime, penicillin G (except strain HG 1178), ciprofloxacin, tetracycline, tobramycin, and the hydroxymetabolite of metronidazole; but they were less susceptible to moxalactam, erythromycin, and metronidazole. The results of the checkerboard titrations are listed in Table 2. All checkerboard titrations showed a variation in the FICI of no more than 0.1 for each strain. The FICIs of all antibiotic combinations listed in Table 2 are the sum of the two lowest fractional inhibitory concentrations determined for each combination per strain. Metronidazole or its hydroxymetabolite combined with cefixime, penicillin G, moxalactam, or ciprofloxacin had FICIs less than or equal to 0.6 (range, from 0.2 to 0.6), indicating synergistic interactions. Synergy did not depend on the susceptibilities of the *A. actinomycetemcomitans* strains to the compounds when the compounds were used separately but was observed for all strains.

The nature of the interaction between metronidazole or its hydroxymetabolite and tetracycline, erythromycin, and tobramycin can be described as additive to indifferent, with FICIs ranging from 0.8 to 2.0.

The results of the clinical pilot study showed that a therapy consisting of mechanical debridement followed by a regimen of ciprofloxacin alone was ineffective in eliminating *A. actinomycetemcomitans* from the periodontal pockets in two patients. The two synergistically acting combinations, metronidazole plus cefixime and metronidazole plus ciprofloxacin, were tested for their ability to eradicate *A. actinomycetemcomitans* from the periodontal lesions in three and four patients, respectively. The results of the pilot study indicated that mechanical therapy followed by treatment

TABLE 1. MICs of metronidazole, its hydroxymetabolite, cefixime, moxalactam, penicillin G, ciprofloxacin, erythromycin, tetracycline, and tobramycin for five *A. actinomycetemcomitans* strains

Antibiotic	MIC (μg/ml) for strain:				
	HG 1174	HG 1175	HG 1176	HG 1177	HG 1178
Metronidazole	40 ± 4	10 ± 1	21 ± 2	15 ± 1	20.0 ± 0.5
Hydroxymetabolite of metronidazole	10 ± 1	3.0 ± 0.3	7.0 ± 0.5	5.0 ± 0.5	5.0 ± 0.5
Cefixime	0.8 ± 0.05	0.6 ± 0.05	0.4 ± 0.05	0.6 ± 0.05	2.2 ± 0.2
Moxalactam	40 ± 4	28 ± 2	33 ± 3	44 ± 4	48 ± 4
Penicillin G	6.5 ± 0.5	3.0 ± 0.2	3.3 ± 0.3	6.0 ± 0.5	20 ± 2
Ciprofloxacin	0.016 ± 0.001	0.012 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.010 ± 0.001
Erythromycin	8.0 ± 0.5	8.0 ± 0.5	4.2 ± 0.3	8.0 ± 0.5	8.0 ± 0.5
Tetracycline	0.5 ± 0.05	0.4 ± 0.02	0.2 ± 0.02	0.4 ± 0.05	0.6 ± 0.05
Tobramycin	2.5 ± 0.5	3.0 ± 0.3	2.0 ± 0.2	3.3 ± 0.3	3 ± 0.3

with either one of the two combinations suppresses *A. actinomycetemcomitans* at the site of infection for at least 3 months.

DISCUSSION

The results of a previous study showed that metronidazole, its hydroxymetabolite, and amoxicillin act synergistically against *A. actinomycetemcomitans* (20). The aim of the present study was to investigate the interactions between metronidazole, its hydroxymetabolite, and a set of antibiotics belonging to different groups by using *A. actinomycetemcomitans* as the test organism. A marked result of the study is that only the β-lactams penicillin G, moxalactam, and cefixime and the 4-fluoroquinolone ciprofloxacin act synergistically with metronidazole and its hydroxymetabolite. Interactions between the hydroxymetabolite and other antibiotics were studied because *A. actinomycetemcomitans* is two to four times more susceptible to this compound than it is to metronidazole and because of its synergistic interaction with metronidazole and amoxicillin (12, 20, 31). Also, its concentrations in human serum, crevicular fluid, and saliva are comparable to those of metronidazole in the same human fluids (10), which indicates that the hydroxymetabolite probably contributes significantly to the effectiveness of the metronidazole-amoxicillin combination. The nature of the synergistic effects between metronidazole, its hydroxymetabolite, and β-lactam antibiotics is still unclear. It is possible that the inhibition of peptidoglycan synthesis by β-lactam antibiotics causes an enhanced influx of metronidazole and its hydroxymetabolite, leading to faster bactericidal activity. We found it striking that synergistic interactions between the β-lactam antibiotics, metronidazole, and its hydroxymetabolite were also present among the strains which were relatively less susceptible to penicillin G (strain HG 1178) and moxalactam (all strains). The synergistic interactions between ciprofloxacin and metronidazole and ciprofloxacin and the hydroxymetabolite of metronidazole are also not understood. The mode of action of ciprofloxacin is inhibition of bacterial DNA gyrase activity (8).

The results of the pilot study on the effects of ciprofloxacin indicate that the high in vitro activity of ciprofloxacin against *A. actinomycetemcomitans* (MIC for 90% of strains tested, 0.010 μg/ml) does not correlate with the in vivo outcome, since a therapy consisting of mechanical debridement followed by a treatment regimen with ciprofloxacin alone was ineffective in eliminating *A. actinomycetemcomitans* from the periodontal pockets. However, the results of the pilot study on the effects of metronidazole plus cefixime and metronidazole plus ciprofloxacin indicate that in vitro synergy between metronidazole, its hydroxymetabolite, and cefixime and ciprofloxacin may have a predictive value for

TABLE 2. FICIs of several antibiotic combinations for five *A. actinomycetemcomitans* strains

Combination[a]	FICI for strain:				
	HG 1174	HG 1175	HG 1176	HG 1177	HG 1178
MET-CEF	0.4	0.6	0.5–0.6	0.2	0.4–0.5
MET-MOX	0.4–0.5	0.2	0.4–0.5	0.3–0.4	0.6
MET-PEN	0.4	0.4–0.5	0.3	0.5	0.5–0.6
MET-CIP	0.4–0.5	0.2–0.3	0.4–0.5	0.5	0.5–0.6
MET-ERY	1.2	0.8–0.9	0.9–1.0	0.9	1.0–1.1
MET-TET	0.8	0.8–0.9	1.1–1.2	0.8–0.9	0.8–1.1
MET-TOB	1.0–1.1	0.8–0.9	1.1–1.2	1.1–1.2	2.0
HYD-CEF	0.5	0.3–0.4	0.5–0.6	0.2–0.3	0.4–0.5
HYD-MOX	0.5–0.6	0.3–0.4	0.6	0.2–0.3	0.4–0.5
HYD-PEN	0.5	0.4	0.4–0.5	0.5	0.5
HYD-CIP	0.3–0.5	0.3–0.4	0.5	0.4–0.5	0.5–0.6
HYD-ERY	1.0–1.1	1.7	0.8–0.9	0.9–1.0	1.0–1.1
HYD-TET	0.8	0.8–0.9	1.1	0.9–1.0	0.8–0.9
HYD-TOB	1.1–1.2	1.1	1.0–1.1	1.0–1.1	2.0

[a] MET, metronidazole; HYD, hydroxymetabolite of metronidazole; CEF, cefixime; PEN, penicillin G; CIP, ciprofloxacin; MOX, moxalactam; TOB, tobramycin; ERY, erythromycin; TET, tetracycline.

the eradication of *A. actinomycetemcomitans* from periodontal lesions. The combination of metronidazole and cefixime might be useful in the treatment of *A. actinomycetemcomitans* periodontitis in patients who develop serious adverse effects when treated with amoxicillin. Cefixime shows only 3 to 7% cross-reactions in penicillin-allergic patients, and cephalosporins are generally well tolerated (1). All *A. actinomycetemcomitans* strains tested were susceptible to cefixime (MIC for 90% of strains tested, 0.8 μg/ml). Metronidazole plus ciprofloxacin can be useful in patients with a history of severe allergic reactions to β-lactam antibiotics.

The results of previous studies (5, 11, 15, 18, 26, 31, 32, 34, 35) and the results of our pilot study suggest that eradication of *A. actinomycetemcomitans* is predictable when combinations of antibiotics show in vitro synergism.

Several studies on treatment strategies for some infectious diseases suggest that they also require that patients be treated with double or even triple antimicrobial combinations rather than a monotherapy. This seems to be true for pelvic inflammatory disease (6) and *Helicobacter pylori*-associated gastritis (19, 22). Also, *A. actinomycetemcomitans*-associated periodontitis and possibly all other infections caused by this microorganism, e.g., endocarditis (9, 13, 14, 22), must be added to the list of infectious diseases that cannot be treated effectively with antimicrobial monotherapies. It is likely that the bacteria involved in such diseases are located in areas where most antibiotics do not reach levels that exceed concentrations greater than the MIC or that the availability of the drug is too low (17). In such cases, therapy mostly fails. However, synergistic interactions between antibiotics can probably solve the problem of low antibiotic concentrations or drug availability at the site of infection. Infective endocarditis caused by *A. actinomycetemcomitans* is mostly treated with antibiotic combinations consisting of a β-lactam combined with either gentamicin or tobramycin (9, 13, 14, 21). The choice of combinations of antibiotics for the treatment of *A. actinomycetemcomitans* infections, such as infective endocarditis, is seldom based on in vitro synergistic interactions between the antibiotics. Moreover, the literature on the in vitro interactions between those antibiotic combinations provides only little information about the nature of the interactions (9, 13, 14, 21). The results of the present and previous studies (20, 34, 35) indicate that synergistically acting antibiotic combinations, i.e., metronidazole plus amoxicillin, metronidazole plus cefixime, and metronidazole plus ciprofloxacin, may be useful in the treatment of *A. actinomycetemcomitans* infections such as *A. actinomycetemcomitans*-associated periodontitis and infective endocarditis. In conclusion, the in vitro synergism between metronidazole, its hydroxymetabolite, and additional antibiotics may be a useful predictor of therapeutic efficacy in the treatment of patients with *A. actinomycetemcomitans*-associated periodontitis and possibly other infections caused by *A. actinomycetemcomitans*.

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