Pharmacodynamic Effects of Antibiotics and Acid Pump Inhibitors on *Helicobacter pylori*

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Pharmacodynamic studies of *Helicobacter pylori* exposed to amoxicillin, clarithromycin, metronidazole, omeprazole, and lansoprazole were performed with microscopy, viable count determination, and bioluminescence assay of intracellular ATP. The pharmacodynamic parameters determined were change in morphology, change in cell density, postantibiotic effect (PAE), and control-related effective regrowth time (CERT). The PAE is delayed regrowth after brief exposure to antibiotics or acid pump inhibitors. CERT was defined as the time required for the bacteria to resume logarithmic growth and return to the pre-exposure inoculum in the test culture minus the corresponding time for the control culture. CERT measures the combined effect of initial killing and PAE. There was a good concordance between the bioluminescence assay and viable counts for determining CERT, which makes this parameter useful for pharmacodynamic studies of the effects of antibiotics and acid pump inhibitors on *H. pylori*. Amoxicillin and metronidazole produced a strong, concentration-dependent initial decrease in CFU per milliliter, but there was a less prominent initial change in intracellular ATP in these cultures. Amoxicillin caused a long PAE when assayed by the bioluminescence assay but no PAE or a negative PAE when assayed by viable count determination. However, amoxicillin showed similar long CERTs with both methods. The pharmacodynamic effects of amoxicillin were concentration dependent up to a maximum response, indicating that concentrations above this level do not increase the antibiotic effect. The PAEs and CERTs of clarithromycin and metronidazole were concentration dependent with no maximum response. With omeprazole and lansoprazole, there was no PAE or CERT.

Duodenal ulcer is strongly associated with *Helicobacter pylori* infections (17). Several reasons for therapeutic failure in the antibiotic treatment of duodenal ulcer caused by *H. pylori* have been proposed and discussed, such as development of resistance to metronidazole (4, 17) and clarithromycin (23) and low MICs and MBCs (12 and 13) have generally been obtained. These parameters are endpoint analyses after a prolonged incubation and represent the final results of a complex interaction between bacteria and a drug (10). MICs and MBCs fail to reveal important pharmacodynamic information about the time course of bacterial responses to antibiotics. To obtain this information, parameters such as initial killing and postantibiotic effect (PAE) (10), effective regrowth time (ERT) (2, 16), and control-related effective regrowth time (CERT) (13, 15, 32) must be studied. PAE describes the delayed regrowth after brief antibiotic exposure. ERT describes the combined effect of the initial killing and PAE. By relating ERT in the drug-exposed culture to ERT in the control culture, we have developed the CERT parameter, which is independent of methods used for bacterial quantitation and determination of antibiotic elimination and growth rate (15). The PAE obtained with different methods sometimes gives conflicting results (10, 16, 24). However, it has been shown that ERT and CERT can circumvent these methodological problems (15, 16).

The aim of this study was to evaluate initial killing, morphological alteration, PAE, and CERT for *H. pylori* after brief exposure to amoxicillin, clarithromycin, metronidazole, omeprazole, and lansoprazole, and to assess whether PAE or CERT is the more reliable parameter for studies of postexposure effects.

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**MATERIALS AND METHODS**

**Bacterial strain.** *H. pylori* NCTC 11637 was used.

**Antibiotics and acid pump inhibitors.** Amoxicillin and metronidazole were purchased from Sigma Chemical Co., St. Louis, Mo., clarithromycin was kindly provided by Abbott Laboratories, Chicago, Ill., omeprazole was from Haslde AB, Malmö, Sweden, and lansoprazole was from Lederle Laboratories, Wayne, N.J. The acid pump inhibitors omeprazole and lansoprazole were acid activated by incubation in a stock solution of 0.1 M citrate-phosphate buffer (pH 5) for 20 min. The following concentrations were tested: amoxicillin, 0.004 to 4 µg/ml; clarithromycin, 0.004 to 4 µg/ml; lansoprazole, 0.5 to 4 µg/ml; metronidazole, 0.12 to 4 µg/ml; omeprazole, 0.1 and 2 to 16 µg/ml.

**Growth medium.** Mueller-Hinton broth (MHB; Gibco Limited, Renfrewshire, Scotland) supplemented with 50-mg/liter CaCl2, 25-mg/liter MgCl2, and 1% fetal calf serum was used as the growth medium.

**MIC determination.** Serial twofold dilutions of antibiotics were prepared in growth medium, and 0.5-ml samples of these dilutions were added to series of test tubes. Bacteria in logarithmic phase were diluted to approximately 2 × 108 CFU/ml, and 0.5-ml aliquots of these cultures were added to the test tubes, which were incubated at 37°C under microaerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) in an incubator box (ASSAB with CO2 and O2 regulator; Kebo Biomed, Spånga, Sweden). Visible growth was recorded after 5 days. MICs were obtained after 3 days. MBCs were obtained three times.

**Viable count.** Viable counts were determined as CFU per milliliter by plating after serial dilution, performed on blood agar plates (Columbia agar base; BBL, Becton Dickinson Company, Cockeysville, Md.) containing 5.9% defibrinated horse blood. The agar plates were incubated for 7 days at 37°C under microaerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) in an incubator box (ASSAB with a CO2 and O2 regulator; Kebo Biomed, Spånga, Sweden). Visible growth was recorded after 5 days. MBCs were obtained after 3 days.

**Bioluminescence assay of intracellular ATP.** A 180-µl sample from the bacterial culture was incubated at 37°C for 10 min with 100 µl of the ATP-hydrolyzing enzyme apyrase, purified grade I (Sigma Chemical Co.) in supplemented

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MHBI, to eliminate extracellular ATP. A 50-μl sample of the apyrase-treated sample was pipetted into 500 μl of boiling 0.1 M Tris buffer, pH 7.75, containing 2 mM EDTA to release the intracellular ATP and inactivate the apyrase. After being heated for 90 s, the extracts were cooled before the assay of intracellular ATP. This extraction was performed in an LKB Biocal 2030 incubator (LKB Products, Bromma, Sweden). Luciferase reagent (100 μl) was added to 550 μl of each cooled extract, and the light intensity was measured in a 1250 Luminometer (LKB Wallac, Turku, Finland) and recorded on a 1250 Display (LKB Wallac). ATP monitoring reagent (Bio Orbit, Turku, Finland) was used in the assay of ATP. Sample ATP levels were calculated by using assays of standard amounts of ATP as a reference. A correction was made for background luminescence. Known amounts of ATP added to the extracts were used as internal standards to correct for inhibition of the luciferase reaction by the reagents. The coefficient of variation for the bioluminescence assay has been shown to vary between 1.7 and 6.5% (31).

**Assay procedure.** Samples were taken for viable count determination and bioluminescence assay of intracellular bacterial ATP. As shown by Thore et al. (33) and Molin et al. (27), 10^-6 M ATP corresponded to approximately 10^8 bacteria/ml. From a culture with bacteria in log phase diluted to 2 × 10^3 CFU/ml, 0.5 ml was transferred to test tubes containing 0.5 ml of antibiotics or acid pump inhibitors in different concentrations. Bacteria were exposed to amoxicillin (2 h), clarithromycin (5 h), metronidazole (8 h), omeprazole (2 h), and lanzoprazole (2 h). The incubation times and drug concentrations were chosen according to half-life and the clinically achieved concentrations of amoxicillin (34), clarithromycin (9), metronidazole (25), omeprazole (8), and lanzoprazole (18). The tubes were incubated at 37°C under microaerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) in the incubator box (ASSAB with a CO2 and O2 regulator). The experiments were performed three times.

**Determination of PAE.** The PAE was calculated from the regrowth curves by using the equation PAE = T – C, where T is the time required for the bacterial population in the control culture to increase 1,000-fold after dilution, and C is the time required for the bacterial population in the control culture to increase 1,000-fold after dilution of the drug and inactivation of the apyrase. Regression analysis was performed to calculate the time to reach 10^-3 CFU/ml. The assay was performed three times.

**Determination of CERT.** The antibiotic-exposed cultures were diluted 1,000 times in MHBI to remove the agents, and in some experiments, beta-lactamase was used for elimination of amoxicillin. This is described in detail in the section on PAE determination. The CERT was calculated from the equation CERT = T – C, where T is the time required for the bacteria to resume logarithmic growth and return to the pre-exposure inoculum and C is the corresponding time for the control culture to return to the pre-exposure inoculum after dilution.

**RESULTS**

**Correlation between bioluminescence assay and viable counting.** In growing cultures of *H. pylori* there was a good correlation between bioluminescence and viable counting for determination of cell numbers (Fig. 3).

**MICs.** The MICs for *H. pylori* NCTC 11637 were as follows: amoxicillin, 0.03 μg/ml; clarithromycin, 0.008 μg/ml; metronidazole, 1 μg/ml; omeprazole, 8 μg/ml.

**Killing curves.** After a 2-h exposure, amoxicillin caused a strong initial decrease in the number of CFU of *H. pylori* per milliliter of culture (Fig. 4A) but no decrease in intracellular ATP. Clarithromycin did not cause any initial decrease in CFU per milliliter (Fig. 4B) or intracellular ATP in *H. pylori* cultures after 5 h of exposure. Metronidazole caused a strong initial decrease in the number of CFU of *H. pylori* per milliliter of culture after 8 h of exposure (Fig. 4C) but no decrease in intracellular ATP. Omeprazole and lanzoprazole did not cause any decrease in CFU per milliliter (Fig. 4D and E) or intracellular ATP after 2 h of exposure.

**PAE and CERT.** After elimination of amoxicillin by dilution, the drug caused a long PAE as determined by bioluminescence assay but no PAE or a negative PAE as determined by viable

**FIG. 1.** Determination of the initial change in cell density and the PAE (A) and CERT (B) of *H. pylori* after exposure to amoxicillin by the viable count method.
counting (Fig. 5). However, amoxicillin caused similar long CERTs when both methods were used (Fig. 1B, 2B, and 5), in contrast to the method-dependent PAE. PAE and CERT determinations after drug elimination with beta-lactamase without dilution gave results corresponding to those obtained with a $10^{-3}$ dilution when the bioluminescence assay and viable counting methods were used. Clarithromycin induced equally long PAEs and CERTs when assayed by the bioluminescence assay and viable counting (Fig. 6). Similar long PAEs and CERTs were induced by metronidazole when assayed by the bioluminescence assay and viable counting (Fig. 7). With omeprazole and lansoprazole, there was no PAE or CERT for *H. pylori* (Fig. 8).

**Microscopy.** After 2 h of exposure of *H. pylori* to amoxicillin, microscopy showed a concentration-dependent induction of spheroplasts with 25 to 40% spheroplasts at the highest concentrations tested (0.03 to 4 μg/ml). To study bacterial morphology after drug elimination, amoxicillin was inactivated by beta-lactamase. During the postexposure phase, the conversion to spheroplasts continued and 4 h after antibiotic inactivation, 90 to 100% of the cells were spheroplasts at all concentrations except the lowest concentrations tested (0.0075 to 0.004 μg/ml). After 60 h, only bacillary forms were present in these cultures. The growth rate between 2 and 60 h was faster in the amoxicillin-exposed culture after drug elimination than in the control culture when the viable count method was used. This caused a negative PAE, and this was also seen when the drug was eliminated by dilution (Fig. 1A). Only bacillary forms of *H. pylori* were seen after exposure to lansoprazole (2 h), omeprazole (2 h), clarithromycin (5 h), and metronidazole (8 h).

**FIG. 2.** Determination of the initial change in cell density and the PAE (A) and CERT (B) of *H. pylori* after exposure to amoxicillin by the bioluminescence assay method.

**FIG. 3.** Correlation between the bioluminescence assay and viable count methods in control cultures of *H. pylori* NCTC 11637.

**FIG. 4.** Initial changes in viability caused by amoxicillin at 0.004 to 4 μg/ml (A), clarithromycin at 0.001 to 1 μg/ml (B), metronidazole at 0.125 to 4 μg/ml (C), lansoprazole at 0.5 to 4 μg/ml (D), and omeprazole at 0.1 and 2 to 16 μg/ml (E).
DISCUSSION

The good correlation in this study between the bioluminescence assay, viable count determination, and microscopy for determination of cell numbers (Fig. 3) (30) is consistent with previous studies including bacterial species other than *H. pylori* (15, 27, 33).

The strong initial bactericidal effect of amoxicillin and metronidazole on *H. pylori* shown in this study (Fig. 4A and C) is in agreement with other studies (1, 5). The lack of a bactericidal effect of clarithromycin (Fig. 4B) is not in accord with the data of Flamm et al. (11). This may be due to their longer exposure time of 8 h compared to the 5 h used in our study. The antibiotics caused no initial decrease in intracellular ATP in *H. pylori* cultures.

Normally, the PAE is calculated on the basis of the time taken for the bacterial numbers to increase 10-fold. However, the ATP values and numbers of CFU per milliliter after 10-fold growth were below the detection limit, as we used a $10^{-3}$ dilution. We therefore used the time required for bacterial numbers, as measured by ATP assay and numbers of CFU per milliliter, to increase 1,000-fold, which we considered adequate since the growth curves for drug-treated and untreated bacteria were parallel once the growth had started. Thus, when PAE calculation is performed during exponential growth there is no difference whether PAE is determined by using a 10- or 1,000-fold increase in bacterial growth. All of the antibiotics caused long PAEs when assayed by bioluminescence (Fig. 5–7). When the viable count method only was used, clarithromycin and metronidazole caused long PAEs (Fig. 6 and 7) while amoxicillin caused no PAE or a negative PAE by the viable count method (Fig. 5). The poor correlation between these methods for assessment of the PAE of amoxicillin on *H. pylori* is due to the stronger initial decrease in the viable count compared to the less prominent initial change in cell density measured by the bioluminescence assay (Fig. 5). Since this initial change in cell density is the starting value for the PAE calculation, these discrepancies jeopardize the possibility of comparing PAE results obtained with different methods (10, 15, 16, 24). CERT, which describes the combined effect of the initial killing during antibiotic exposure and the PAE, can circumvent these methodological differences (15). Amoxicillin, clarithromycin, and metronidazole caused similar long CERTs as determined by both viable counting and the bioluminescence assay (Fig. 5–7). This indicates that CERT is independent of the method used for bacterial quantitation. Furthermore, this study showed equally long CERTs for amoxicillin and *H. pylori* when amoxicillin was eliminated by dilution or beta-lactamase inactivation, which is in agreement with a previous study (15).

The faster regrowth determined by the viable count method after antibiotic elimination in the amoxicillin-exposed culture of *H. pylori* compared to the control culture may be an overestimation of the initial decrease in the cell density measured by the viable count method (Fig. 1A). This leads to an underestimation of the length of the PAE, which was, in fact, negative for amoxicillin and *H. pylori* when measured by the viable count method (Fig. 1A and 5). This can be explained by the inability of amoxicillin-induced spheroplasts to survive on agar plates, although these cells probably repair their cell walls in a broth milieu and resume faster growth in the amoxicillin-exposed culture than in the control culture when measured by the
viable count method (Fig. 1). It must be emphasized that different techniques for determining killing and regrowth of bacteria measure different bacterial activities. The viable count method is an indirect method, as it includes overnight incubation on agar plates. In contrast, the bioluminescence assay and microscopy directly measure bacterial density directly in the culture. This influences the length of the PAE. It has been claimed that the bioluminescence assay overestimates the bacterial density after antibiotic exposure since it may measure intracellular ATP from dead cells (10). The morphological change in H. pylori toward spheroplasts (28) or coccoid cells (1, 5, 6, 28) that is induced by antibiotics has also been documented in other studies. When discussing the morphological changes, it is probably important to differentiate between spheroplasts induced by betalactam antibiotics, which can revert to bacillary forms (Fig. 1), and coccoid forms induced by erythromycin and bismuth subsalicylate (28), as well as by nutrient deprivation (35) and extended incubation (30). Only Nilius et al. (28) have distinguished between coccoid cells and beta-lactam-induced spheroplasts, and it is important to investigate whether beta-lactam-induced spheroplasts differ from coccoid cells induced by other drugs and environmental stress in terms of cell wall damage and the ability to revert to ordinary bacillary forms. We do not know the biological significance of these coccoid forms and spheroplasts. In a study of the morphological conversion of H. pylori from the bacillary to the coccoid form during prolonged incubation, a 1,000-fold lower ATP level was found in coccoid cells than in the bacillary form, indicating low metabolic activity in the coccoid cells (30). We were not able to demonstrate conversion from the coccoid to the bacillary form in the previous study (30). In contrast, the spheroplasts induced by amoxicillin reverted to bacillary forms in broth but not on agar plates in the present study. Metronidazole caused no morphological alteration of H. pylori after 8 h of incubation. This was confirmed by Armstrong et al. (1), who found no structural changes in the presence of metronidazole. The difference in the metronidazole PAE on H. pylori obtained with the viable count and bioluminescence assay methods was not as pronounced as for amoxicillin and H. pylori, and no negative PAE of metronidazole was seen (Fig. 7). The small difference between the PAEs of metronidazole obtained with the viable count and bioluminescence assay methods is probably due to the fact that partially damaged H. pylori bacillary forms may survive in broth but not on agar plates.

Brief exposure of H. pylori to acid pump inhibitors (2 h) or clarithromycin (5 h) showed no morphological alteration in our study. Nilius et al. (28), in contrast to us, showed coccoid forms of H. pylori after exposure to erythromycin, but this was after a very long exposure.

The dose regimen used for treatment of H. pylori with antibiotics is based on dose regimens for other bacterial infections. These bacteria have much shorter generation times than H. pylori, which makes pharmacodynamic studies of H. pylori warranted. Based on clinical studies (3, 22), the trends in the antibiotic treatment of H. pylori to minimize the side effects and get better compliance and to improve the eradication rate involve lower doses with longer intervals between the doses and shorter courses.

In our study, metronidazole and clarithromycin caused long, concentration-dependent PAEs and CERTs without a maximum response, indicating that high doses may be preferable (Fig. 6–8). High doses of these drugs may prevent development of resistance of H. pylori to metronidazole and clarithromycin, which has been shown for other antibiotics and bacterial species (29). The PAE and CERT of amoxicillin were concentration-dependent up to a maximum response, indicating that concentrations above this level do not improve the antibiotic effect (Fig. 5 and 8).

No PAE, CERT, or initial decrease in viable count or intracellular ATP level was shown for H. pylori during short exposure to physiological concentrations of acid pump inhibitors (Fig. 4D and E and 8). The absence of an antibacterial effect of physiological concentrations of the acid pump inhibitors used in our study is in agreement with previous in vitro (19) and clinical (22) results obtained with only omeprazole. This absence of delayed regrowth of H. pylori after elimination of acid pump inhibitors shown in the present study (Fig. 8) is in agreement with the results from two pretreatment studies with omeprazole before combination treatment. Koelz et al. (20) reported that in a double-blind randomized trial omeprazole treatment for 2 weeks prior to amoxicillin and omeprazole treatment did not affect the eradication rate of H. pylori. Caviglia et al. (7) found similar results when they showed that 2 weeks of pretreatment with omeprazole prior to combination treatment with amoxicillin and omeprazole did not impair the rate of H. pylori eradication. This is in conflict with a study by Labens et al. (21) using amoxicillin and omeprazole. They found in a multivariate statistical analysis of 405 patients in 11 studies that pretreatment with omeprazole was a significant independent factor in treatment failure.

In conclusion, the good concordance between the biolumi-
nessance assay and viable count determination for determining CERT makes this parameter most reliable for postexposure studies of the effects of antibiotics and acid pump inhibitors on *H. pylori*. The pharmacodynamic effects of amoxicillin were concentration dependent up to a maximum response, indicating that concentrations above this level do not increase the antibiotic effect. The PAE and CERT of clarithromycin and metronidazole were concentration dependent with no maximum response. With omeprazole and lansoprazole, there was no PAE or CERT.

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