Risk of Development of In Vitro Resistance to Amoxicillin, Clarithromycin, and Metronidazole in Helicobacter pylori

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We have studied initial killing, morphological alterations, the frequency of occurrence, and the selective growth of resistant subpopulations of Helicobacter pylori during exposure to amoxicillin, clarithromycin, or metronidazole by bioluminescence assay of intracellular ATP levels, microscopy, and a viable count assay. We found an induction of spheroplasts and a decrease in intracellular ATP levels after 21 h of exposure to high concentrations of amoxicillin. During clarithromycin exposure the onset of a decrease in intracellular ATP levels started after prolonged incubation, and with the highest concentration of clarithromycin an induction of coccoid forms was seen after 68 h. Metronidazole exposure resulted in the strongest initial decrease in intracellular ATP levels, and coccoid forms were seen after 21 h of exposure to high concentrations of metronidazole. Amoxicillin caused a low-level increase in resistant subpopulations, which indicates a need for surveillance of the amoxicillin susceptibility of H. pylori in order to detect decreasing susceptibility. No increase in the numbers of resistant subpopulations was demonstrated during clarithromycin exposure. Metronidazole selected resistant subpopulations, which caused high-level resistance in H. pylori.

Helicobacter pylori infection is a principal cause of chronic gastritis type B (13) and is associated with gastric cancer (19, 20, 33). Eradication of H. pylori prevents relapse of duodenal ulcer, and treatment of this infection has now become standard for patients with peptic ulcer disease (22, 37). The regimen most widely used today to eradicate H. pylori is combination therapy with two antibiotics and bismuth (17, 27) or an acid pump inhibitor (3, 22). A major reason for

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by the number of colonies on plates without antibiotic. The experiments were repeated three times. Ten passages of the metronidazole-resistant cultures were done in drug-free MHB. Susceptibility testing on agar plates was done with passed cultures in antibiotic-free broth by the E-test (AB Biodisk).

**Determination of antibiotic concentrations in H. pylori cultures.** Samples from *H. pylori* cultures exposed to 0.25 \( \mu \)g of amoxicillin per ml, 0.25 \( \mu \)g of clarithromycin per ml, or 32 \( \mu \)g of metronidazole per ml were taken after 0, 21, 46, 68, 96, 118, 142, 166, 191, 214, and 267 h incubation at 37\(^\circ\)C under microaerobic conditions. The samples were put in wells of a PDM agar (AB Biodisk) tray with *Micrococcus luteus* ATCC 9341 for determination of amoxicillin and clarithromycin concentrations and in wells of a PDM agar plus 5% defibrinated horse blood (AB Biodisk, Solna, Sweden) tray with *Clostridium perfringens* ATCC 13124 for determination of metronidazole concentration. The trays with *M. luteus* were incubated overnight under aerobic conditions, and the trays with *C. perfringens* were incubated overnight under anaerobic conditions. The resulting inhibition zones surrounding the wells were measured and were compared with those obtained by linear regression analysis with standard concentrations of drugs.

**RESULTS**

**Growth and morphology of H. pylori during exposure to amoxicillin.** Growth of the cultures with an inoculum of 1.9 \( \times \) \( 10^8 \) M ATP was monitored, and three growth patterns were seen. Intracellular ATP levels increased in the cultures exposed to low concentrations of amoxicillin (\( \leq 0.004 \) \( \mu \)g/ml) (Fig. 1). There was an initial growth inhibition in the cultures exposed to 0.008 \( \mu \)g of amoxicillin per ml (Fig. 1). The cultures exposed to \( \geq 0.015 \) \( \mu \)g of amoxicillin per ml showed an initial decrease in intracellular ATP levels after 46 h (Fig. 1). Microscopy showed spheroplasts in cultures in which a decrease in intracellular ATP levels occurred. After 118 h a few bacillary forms were seen, and the numbers of these forms increased when the intracellular ATP level in these cultures increased. There was no decrease in the amoxicillin concentration in the broth cultures containing 0.008 and 0.015 \( \mu \)g of amoxicillin per ml when regrowth occurred. In the culture exposed to 0.03 \( \mu \)g of amoxicillin per ml, intracellular ATP levels increased after 191 h, and microscopy showed a mixed population of bacillary forms, spheroplasts, and coccoid cells. In this culture there was a reduction in the amoxicillin concentration, and at 191 h, when the culture regrew, only 50% of the initial amoxicillin concentration was left in the broth. At concentrations above 0.03 \( \mu \)g of amoxicillin per ml there was no regrowth.

**Population analyses of cultures exposed to amoxicillin.** Population analyses were performed with the unexposed cultures and cultures exposed to amoxicillin at concentrations of 0.008, 0.015, and 0.03 \( \mu \)g/ml. The bacteria in the unexposed cultures were resistant to up to 0.008 \( \mu \)g/ml (Fig. 2). At higher concentrations on agar plates there was a reduction in the frequency of resistant variants for the unexposed broth cultures (Fig. 2). The cultures exposed to 0.008 and 0.015 \( \mu \)g of amoxicillin per ml in broth were resistant to up to 0.015 \( \mu \)g/ml, and the frequency of resistant variants exposed to amoxicillin at 0.03 \( \mu \)g/ml was \( 10^{-1} \) (Fig. 2). The frequency of resistant variants in the cultures exposed to 0.03 \( \mu \)g of amoxicillin per ml was lower than that for the unexposed cultures (Fig. 2). Population analyses were performed with the control cultures on two different occasions with an interval of 3 days, and no reduction in the amoxicillin concentrations on the agar plates was found.

**Growth and morphology of H. pylori during exposure to clarithromycin.** Growth of the cultures with an inoculum of 2.3 \( \times \) \( 10^8 \) M ATP was monitored. Intracellular ATP levels increased in the cultures exposed to low concentrations of clarithromycin (\( \leq 0.06 \) \( \mu \)g/ml) (Fig. 3). There was a concentration-dependent inhibition of the increase in intracellular ATP levels, and after prolonged incubation there was a concentration-dependent decrease in intracellular ATP levels in the cultures (Fig. 3). Microscopy showed bacillary forms in the growing cultures, and a conversion from bacillary to coccoid forms was seen when the intracellular ATP levels decreased. No cultures showed regrowth. The clarithromycin concentrations in broth were stable throughout the experiment.

**Population analyses of cultures exposed to clarithromycin.** Population analyses were performed with the unexposed cultures and cultures exposed to clarithromycin at concentrations of 0.015 and 0.008 \( \mu \)g/ml. The unexposed cultures and the cultures exposed to 0.008 \( \mu \)g of clarithromycin per ml were resistant to up to 0.015 \( \mu \)g/ml (Fig. 4). At higher concentrations there was a reduction in the frequency of resistant variants to \( 10^{-1} \) after exposure to clarithromycin at 0.03 \( \mu \)g/ml (Fig. 4). For cultures exposed to 0.015 \( \mu \)g of clarithromycin per ml the frequency of resistant variants was lower after exposure...
to clarithromycin at 0.015 and 0.03 μg/ml than that for the unexposed cultures and the cultures exposed to 0.008 μg of clarithromycin per ml (Fig. 4). Population analyses were performed with the control cultures on two different occasions with an interval of 3 days, and no reduction in the clarithromycin concentration in the agar plates was found.

**Growth and morphology of H. pylori during exposure to metronidazole.** Growth of the cultures with an inoculum of 1.9 × 10^8 M ATP was monitored, and two growth patterns were seen. Intracellular ATP levels increased in the cultures exposed to low concentrations of metronidazole (≤0.5 μg/ml) (Fig. 5). The cultures exposed to 1 to 32 μg of metronidazole per ml showed an initial decrease in intracellular ATP levels (Fig. 5), and microscopy showed a conversion from bacillary to coccoid forms after 21 h. When the intracellular ATP levels increased in the cultures exposed to 1 to 4 μg of metronidazole per ml, a change in morphology was seen by microscopy, from coccoid forms to bacillary forms. In cultures with concentrations above 4 μg of metronidazole per ml there was no regrowth (Fig. 5). The metronidazole concentrations in broth were stable throughout the experiment.

**Population analyses of cultures exposed to metronidazole.** Population analyses were performed with the unexposed cultures and cultures exposed to metronidazole at concentrations of 1 to 4 μg/ml. The bacteria in the unexposed cultures were resistant to up to 0.25 μg/ml (Fig. 6). With higher concentrations there was a reduction in the frequency of resistant variants for the unexposed cultures (Fig. 6). For the cultures ex-
posed to 1 to 4 µg of metronidazole per ml, resistant variants were resistant to up to 32 µg/ml (Fig. 6). The selection of resistant variants was concentration dependent, and after exposure to the highest concentration (4 µg of metronidazole per ml) all bacteria in the population were resistant to metronidazole at 32 µg/ml (Fig. 6). Population analyses were performed with the control cultures on three different occasions with a total interval of 13 days, and no reduction in the metronidazole concentrations on the agar plates was found (Fig. 6). The resistance remained stable through 10 passages in MHB for all cultures in which metronidazole resistance developed.

**DISCUSSION**

This study showed an initial decrease in intracellular ATP levels during exposure of *H. pylori* to high concentrations of amoxicillin (Fig. 1), and this bactericidal effect of amoxicillin is in agreement with the effect found in a study by Berry et al. (6). During exposure of *H. pylori* to amoxicillin, microscopy showed spheroplasts after 21 h, which is in accordance with the findings of a study by Nilius et al. (32) and a previous study by us (40), in which we found a concentration-dependent induction of spheroplasts after only 2 h. Other investigators have reported a morphologic conversion of *H. pylori* during exposure to...
amoxicillin but have not distinguished between coccoïd forms and spheroplasts (6, 7). Armstrong et al. (1) reported central clearing and vesiculation of *H. pylori* after a 24-h exposure to amoxicillin but did not discuss these findings in terms of coccoïd forms or spheroplasts. No reports on the clinical significance of amoxicillin resistance in *H. pylori* have been published, and Glupczynski et al. (16) found an unchanged susceptibility of *H. pylori* to amoxicillin over a 5-year period. We found a small increase in the numbers of resistant subpopulations in all except one of the regrowing cultures exposed to amoxicillin. In one culture (containing 0.03 μg of amoxicillin per ml), regrowth occurred after 191 h due to a decrease in the concentration of amoxicillin in the broth (Fig. 2). Our results are in agreement with those of Haas et al. (18), who found increased amoxicillin MICs during exposure to amoxicillin in several passages. Our results and those of Haas et al. (18) indicate that there is a need for surveillance of the amoxicillin susceptibility of *H. pylori* in order to detect decreasing levels of susceptibility.

After a prolonged incubation, clarithromycin exposure resulted in a concentration-dependent decrease in intracellular ATP levels in *H. pylori* cultures (Fig. 3). Similar results were found by Flamm et al. (15), who demonstrated a bactericidal effect of clarithromycin on *H. pylori* after 8 h of exposure to clarithromycin. In an earlier study (40) we could not find any bactericidal effect after 5 h of exposure of *H. pylori* to clarithromycin, but this was probably due to a shorter exposure time. A conversion from bacillary to coccoïd forms was seen by microscopy. This change is probably due to a low frequency of occurrence of resistant subpopulations rather than a conversion from coccoïd to bacillary forms. This caused a decreased susceptibility to metronidazole, which is in agreement with the findings of Haas et al. (18), who reported an increase in the MIC of metronidazole for *H. pylori* after several passages during exposure to metronidazole. When evaluating the development of resistance to metronidazole during treatment, it is important to study whether there is a selection of spontaneous resistant variants of the infecting strain or whether there is reinfection with an exogenous strain (23, 35). The metronidazole resistance in our study was stable during 10 passages, which is in agreement with the findings of a study by Haas et al. (18), but there have also been reports of unstable metronidazole resistance. Zwet et al. (48) found that metronidazole resistance induced in vitro was reversed in 30% of the isolates by further culture on antibiotic-free plates. The different results concerning the stability of metronidazole resistance might be explained by methodological differences such as the use of

![Population analysis of H. pylori in an unexposed cultures (■, □, and ▲) and cultures exposed to metronidazole at 1 to 4 μg/ml (○, 1 μg/ml; +, 2 μg/ml; ●, 4 μg/ml).](http://aac.asm.org/)

**FIG. 6.** Population analysis of *H. pylori* in an unexposed cultures (■, □, and ▲) and cultures exposed to metronidazole at 1 to 4 μg/ml (○, 1 μg/ml; +, 2 μg/ml; ●, 4 μg/ml).
different inocula (21) and by the use of different incubation conditions (8, 38, 47).

When studying the morphology of _H. pylori_ it is important to differentiate between coccoïd cells (1, 6) and spheroplasts (32). In our earlier study we found a rapid induction of spheroplasts during exposure of _H. pylori_ to amoxicillin (40), while the rate of conversion to coccoïd forms during exposure to clarithromycin and metronidazole in this study was slower. The spheroplasts are larger than the coccoïd forms seen by microscopy (unpublished results). The coccoïd forms changed color, from orange to green, during prolonged exposure to clarithromycin and metronidazole when acridine orange staining was used (unpublished results). A cell containing more RNA than DNA stains orange, and a cell in which much of the RNA has been degraded but still retains its DNA stains green (24). The degradation of RNA has been correlated with a loss of viability (10). The spheroplasts stained orange (unpublished results), and we found in an earlier study (40) that they reverted to bacillary forms. In another previous study (39) we found a low ATP level in the coccoïd cell during prolonged incubation of _H. pylori_, but we were not able to demonstrate a conversion to bacillary forms.

In conclusion, we found an induction of spheroplasts and a decrease in intracellular ATP levels after 21 h of exposure of _H. pylori_ to high concentrations of amoxicillin. During clarithromycin exposure the onset of the decrease in intracellular ATP levels started after prolonged incubation, and with the highest concentration of clarithromycin an induction of coccoïd forms was seen after 68 h. Metronidazole exposure resulted in the strongest initial decrease in intracellular ATP levels, and coccoïd forms were seen after 21 h of exposure to high concentrations of metronidazole. Amoxicillin caused a low-level increase in the numbers of resistant subpopulations, which indicates that there is a need for surveillance of the amoxicillin susceptibility of _H. pylori_ in order to detect decreasing susceptibility. No increase in resistant subpopulations was demonstrated during clarithromycin exposure. Metronidazole selected resistant subpopulations, which caused high-level resistance in _H. pylori_.

REFERENCES


