Triple therapy with bismuth subcitrate and two antimicrobial agents, including metronidazole, is the most effective treatment regimen for eradication of *Helicobacter pylori* infections (7, 21). The results of standard in vitro metronidazole susceptibility tests have proven to be of value in predicting the outcome of therapy regimens in which metronidazole is used (19, 22). Nevertheless, in studies in which triple therapy is given for 2 weeks or more, eradication in the group of patients with pretreatment resistant strains is still up to 68% (1, 4, 17, 19, 22), which is generally higher than the success rate of dual therapy (17, 18). This suggests that metronidazole still shows some activity against metronidazole-resistant *H. pylori* strains.

Determination of susceptibility in *H. pylori* is performed under microaerophilic conditions, because *H. pylori* grows best under these circumstances. Anaerobic incubation, however, can alter the resistance of *H. pylori* strains to metronidazole (5). Moreover, in a previous study, we have shown that metronidazole resistance induced in vitro could disappear after prolonged microaerophilic culture (23).

In this study, we focused on *H. pylori* strains, obtained from antral biopsy samples, with primary metronidazole resistance. We looked at the stability of the resistance of these strains under different conditions in order to gain insight in the observed discrepancy between in vitro susceptibility test results and the eradication results in vivo.

Different methods have been evaluated to determine the susceptibility of *H. pylori* to metronidazole. Both disk diffusion and agar dilution have been used for this purpose (8, 16, 17, 19, 22). However, since its introduction in 1988, the E test has been shown to be especially useful when studying slowly growing bacteria with fastidious growth requirements (3). Because this rather simple method is very accurate for determining the MIC for *H. pylori*, it was used in this study (6, 11, 14).

Culture of antral biopsy samples was performed under microaerophilic conditions (Campy-PAK Systems; BBL Microbiology Systems, Cockeysville, Md.) with Belo-horizontium medium containing brain heart infusion agar (35 g/l), sheep blood (10%), nalidixic acid (20 mg/liter), vancomycin (6 mg/liter), amphotericin B (2 mg/liter), and 2,3,5-triphenyltetrazolium chloride (40 mg/liter). Incubation was done at 36°C. The susceptibility test was performed by inoculating the culture with an E test (Anaerocult A; Merck, Darmstadt, Germany) for 48 h at 36°C. After this, the MIC was determined by a microdilution method in blood agar plates and by positive biochemical tests (oxidase, catalase, and urease).

After the determination of the MIC, three successive subcultures were performed on antibiotic-free blood agar base (Oxoid CM 55). This was followed by a redetermination of the MIC to assess the stability of the resistance. For this final determination, an inoculum size the same as that for the initial MIC determination was used. In addition, in seven primary resistant strains, the effect of temporary anaerobic incubation on metronidazole susceptibility was studied as follows. Suspensions of strains were swabbed out on blood agar base and incubated under strict anaerobic conditions in the presence of an E test strip (Anaerocult A; Merck, Darmstadt, Germany) for 24 h at 36°C. Afterwards, further incubation of these blood agar plates took place under microaerophilic conditions (Campy-PAK) for another 3 days. After this period, MICs were registered. The same procedure of anaerobic incubation of resistant strains for 24 h was done on blood agar plates without the application of the E test strip. The strip was only applied just before the subsequent 3 days of microaerophilic incubation of the plates. After this period of incubation, MICs were recorded. Finally, the E test strip was applied to plain blood agar plates, and anaerobic incubation took place without the addition of the *H. pylori* suspension. On this occasion, the *H. pylori* suspension was made after incubation for 24 h under anaerobic conditions and the plates were inoculated just before the start of the microaerophilic incubation period of 3 days.
In 13 of the 17 primary resistant isolates, the MIC remained at >32 μg/ml after three successive subcultures on antibiotic-free medium. In four other H. pylori strains, however, the MIC was in the range of susceptibility (MIC, <8 μg/ml) after three subcultures.

In the strains that had been incubated under anaerobic conditions together with the E test strip, the MIC diminished to values in the range of susceptibility in all cases. After subculture and subsequent incubation under microaerophilic conditions, the MIC was again >32 μg/ml. Anaerobic incubation of H. pylori in the absence of a metronidazole E test strip did not yield a change in the MIC after subsequent microaerophilic incubation. There was also no alteration in the MIC when the E test strip was first incubated under anaerobic conditions and the primary resistant strain was inoculated afterwards (Table 1).

H. pylori rapidly develops metronidazole resistance both in vitro and in vivo (12, 13, 19, 22). Still, little information is available concerning the mechanism of metronidazole resistance. Recently, however, it has been demonstrated that H. pylori can acquire resistance by natural transformation (24).

When triple therapy is given to patients harboring resistant strains, eradication can still be obtained in a considerable number of them (1, 17, 19, 22). Especially, triple therapy of longer duration may overcome therapy failure due to metronidazole resistance (4). Noach et al. (17) demonstrated that eradication could be obtained in 68% of patients harboring metronidazole-resistant isolates when treated for 28 days with a triple-therapy regimen containing metronidazole. In another group, treated with a dual-therapy regimen without metronidazole for 28 days, eradication appeared to be only 37%. These results suggest that even when pretreatment metronidazole resistance is found, addition of the drug can be helpful in obtaining in vivo eradication.

In this study, we demonstrated that the registered in vitro resistance changed considerably according to incubation conditions. First, in 4 of 17 H. pylori isolates (24%), primary resistance appeared to be a nonstable phenomenon. Second, after anaerobic incubation in the presence of metronidazole, resistant strains became susceptible.

In a previous study, metronidazole resistance obtained after serial passage of susceptible H. pylori strains on metronidazole-containing plates was nonstable in 24% (23), similar to the nonstable metronidazole resistance under microaerophilic culture conditions seen in our pretreatment resistant clinical isolates. It is therefore a moot point whether all apparently resistant isolates should be classified as such, because in some of these strains, the observed resistance is not stable.

Recent observations by Rautelin et al. (20) fit well with the hypothesis of the existence of nonstable metronidazole resistance. In a patient Rautelin examined, there were three H. pylori isolates with similar ribopatterns. The first was obtained from a biopsy taken before the start of therapy, the second was obtained from a biopsy taken after the end of therapy, and the third was recovered 9 months later. The first and third isolates appeared to be susceptible to metronidazole, while the second isolate was resistant to the drug. Probably there was a return of resistance of H. pylori toward susceptibility in vivo, suggesting that nonstability of metronidazole resistance is not merely a laboratory phenomenon.

In our studies, resistant strains became susceptible, after a brief period of anaerobic incubation, only when the anaerobic incubation was performed in the presence of a metronidazole E test. Anaerobic incubation of a strain without a strip or incubation of a strip and inoculation of the strain after the anaerobic period did not influence the original MIC.

It is likely that only under anaerobic conditions does an interaction between the H. pylori strain and metronidazole occur and that this interaction is essential for the alteration of the MIC afterward. Resistance is restored after subsequent microaerophilic incubation. This means that the anaerobic situation is essential for obtaining susceptibility in initially resistant strains. Our findings support the hypothesis of Cederbrant et al. (5) that resistant strains have lost the ability to achieve a redox potential low enough to reduce the nitro group of metronidazole (5), especially because resistance only disappears when the anaerobic incubation of the H. pylori strain is done in the presence of metronidazole. Under microaerophilic conditions, in susceptible strains, metronidazole enters the cell and reduction of the nitro group to an active metabolite which then reacts with DNA causing strand breaks and subsequent cell death occurs. Under microaerophilic conditions, this pathway is blocked for resistant strains, but a period of anaerobic incubation is able to restore susceptibility.

It has been demonstrated in vitro that, even under microaerophilic conditions, some killing of resistant H. pylori strains by metronidazole occurs (15). Because in that experiment there was no loss of metronidazole as these resistant bacteria were killed, it was suggested that “futile cycling” had occurred (9). In that case, the killing could be the result of the production of the superoxide radical anion and the subsequent accumulation of the toxic hydroxyl radical (10).

In conclusion, we believe that there are probably three explanations for the observed discrepancy between the in vitro resistance of H. pylori to metronidazole and its apparent remaining efficacy against resistant strains in vivo. First, for some strains from clinical isolates, the resistance observed in culture under microaerophilic conditions is evidently nonstable, making the classification of these strains as true metronidazole resistant doubtful. Second, it is possible that the oxygen conditions in the ecological niche of H. pylori alter during a certain period of time and that temporarily anaerobic conditions occur, enhancing the reduction of metronidazole in previously resistant strains with subsequent killing of the bacteria. Third, even in H. pylori strains resistant to metronidazole, some killing of the microorganism can occur as a result of the accumulation of toxic products from futile cycling.

**REFERENCES**
