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Explanations for High Rates of Eradication with Triple Therapy Using Metronidazole in Patients Harboring Metronidazole-Resistant *Helicobacter pylori* Strains

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In 4 of 17 *Helicobacter pylori* strains obtained from antral biopsy samples, the registered primary resistance (MIC, >32 μg/ml) appeared to be nonstable after prolonged microaerophilic incubation. In all resistant strains tested, susceptibility could be obtained when culture under normal microaerophilic conditions was preceded by a period of anaerobic incubation. Both of these findings may explain the observed discrepancy between the results of in vitro susceptibility tests and the eradication obtained in vivo.

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-horizonte medium containing brain heart infusion agar (35 g/ml), 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 plates were examined after 4 and 7 days of incubation. After incubation, typical glossy colonies appeared with golden pigmentation caused by reduction of tetrazolium chloride. Further identification was done by Gram staining of these colonies and by positive biochemical tests (oxidase, catalase, and urease).

Subsequently, MICs were determined by the use of E tests on agar plates containing blood agar base (Oxoid CM 55) with 5% sheep blood. Differences in inoculum size can influence the outcome of susceptibility testing (2); however, when the E test is used in *H. pylori* strains, minor variations in the inoculum size will not alter MIC results (6). In this study, for each strain, plates were inoculated with one loopful of an *H. pylori* suspension adjusted to a turbidity approximating that of a McFarland no. 1 standard.

A total of 17 *H. pylori* strains were examined, with resistance at the primary isolation expressed as an initial MIC of >32 μg/ml in all cases.

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.

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TABLE 1. Effect of different procedures on metronidazole susceptibility in resistant *H. pylori* strains

Procedure	No. of strains tested	MIC (no. of strains)
None	17	>32 (17)
Three microaerophilic subcultures	17	>32 (13) <8 (4)
24-h anaerobic incubation of *H. pylori* and the E test[a]	7[b]	<8 (7)
Subculture (microaerophilic incubation)	3[c]	>32 (3)

[a] When *H. pylori* was incubated in the absence of a metronidazole E test, no change in MIC was observed.
[b] Seven of the 13 strains that remained resistant after three subcultures.
[c] Three of the seven previously resistant strains that had become susceptible under anaerobic incubation.

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, 23). 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

1. **Bell, G. D., K. Powell, S. M. Burridge, A. Pallecaros, P. H. Jones, and P. W. Gant.** 1992. Experience with "triple" anti-*Helicobacter pylori* eradication therapy: side effects and the importance of testing the pre-treatment bacterial isolate for metronidazole resistance. Aliment. Pharmacol. Ther. **6:**427–435.
2. **Berger, S. A., A. Gorea, M. Moskowitz, M. Santo, and T. Gilat.** 1993. Effect

of inoculum size on antimicrobial susceptibility of *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. **12:**782–783.
3. **Bolmström, A., S. Arvidson, M. Ericsson, and Å. Karlsson.** 1988. A novel technique for direct quantification of antimicrobial susceptibility of microorganisms, abstr. 1209, p. 325. *In* Program and abstracts of the 28th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
4. **Burette, A., Y. Glupczynski, and C. De Prez.** 1992. Evaluation of various multi-drug eradication regimens for *Helicobacter pylori*. Eur. J. Gastroenterol. Hepatol. **4:**817–823.
5. **Cederbrant, G., G. Kahlmeter, and A. Ljungh.** 1992. Proposed mechanism for metronidazole resistance in *Helicobacter pylori*. J. Antimicrob. Chemother. **29:**115–120.
6. **Cederbrant, G., G. Kahlmeter, and A. Ljungh.** 1993. The E test for antimicrobial susceptibility testing of *Helicobacter pylori*. J. Antimicrob. Chemother. **31:**65–71.
7. **Chiba, N., J. W. Rademaker, B. V. Rao, and R. H. Hunt.** 1991. Eradication of *Helicobacter pylori*—meta-analysis to determine optimal therapy. Gut **32:** A1220–A1221.
8. **DeCross, A. J., B. J. Marshall, R. W. McCallum, S. R. Hoffman, L. J. Barrett, and R. L. Guerrant.** 1993. Metronidazole susceptibility testing for *Helicobacter pylori*: comparison of disk, broth, and agar dilution methods and their clinical relevance. J. Clin. Microbiol. **31:**1971–1974.
9. **Edwards, D. I.** 1993. Nitroimidazole drugs—action and resistance mechanisms. I. Mechanisms of action. J. Antimicrob. Chemother. **31:**9–20.
10. **Edwards, D. I.** 1993. Nitroimidazole drugs—action and resistance mechanisms. II. Mechanisms of resistance. J. Antimicrob. Chemother. **31:**201–210.
11. **Glupczynski, Y., M. Labbé, W. Hansen, F. Crokaert, and E. Yourassowsky.** 1991. Evaluation of the E test for quantitative antimicrobial susceptibility testing of *Helicobacter pylori*. J. Clin. Microbiol. **29:**2072–2075.
12. **Goodwin, C. S., B. J. Marshall, E. D. Blincow, D. H. Wilson, S. Blackbourn, and M. Phillips.** 1988. Prevention of nitroimidazole resistance in *Campylobacter pylori* by coadministration of colloidal bismuth subcitrate: clinical and in vitro studies. J. Clin. Pathol. **41:**207–210.
13. **Haas, C. E., D. E. Nix, and J. J. Schentag.** 1990. In vitro selection of resistant *Helicobacter pylori*. Antimicrob. Agents Chemother. **34:**1637–1641.
14. **Hirschl, A. M., M. M. Hirschl, and M. L. Rotter.** 1993. Comparison of three methods for the determination of the sensitivity of *Helicobacter pylori* to metronidazole. J. Antimicrob. Chemother. **32:**45–49.
15. **Lacey, S. L., S. F. Moss, and G. W. Taylor.** 1993. Metronidazole uptake by sensitive and resistant isolates of *Helicobacter pylori*. J. Antimicrob. Chemother. **32:**393–400.
16. **Logan, R. P. H., P. A. Gummett, J. J. Misiewicz, Q. N. Karim, M. M. Walker, and J. H. Baron.** 1991. One week eradication regimen for *Helicobacter pylori*. Lancet **338:**1249–1252.
17. **Noach, L. A., M. A. Bertola, M. P. Schwartz, E. A. J. Rauws, and G. N. J. Tytgat.** 1994. Treatment of *Helicobacter pylori* infection: an evaluation of various therapeutic trials. Eur. J. Gastroenterol. Hepatol. **6:**585–591.
18. **O'Riodan, T., E. Mathai, E. Tobui, D. McKenna, C. Keane, and E. Sweeney.** 1990. Adjuvant antibiotic therapy in duodenal ulcers treated with colloidal bismuth subcitrate. Gut **31:**999–1002.
19. **Rautelin, H., K. Seppällä, O.-V. Renkonen, U. Vainio, and T. U. Kosunen.** 1992. Role of metronidazole resistance in therapy of *Helicobacter pylori* infections. Antimicrob. Agents Chemother. **36:**163–166.
20. **Rautelin, H., W. Tee, K. Seppälä, and T. U. Kosunen.** 1994. Ribotyping patterns and emergence of metronidazole resistance in paired clinical samples of *Helicobacter pylori*. J. Clin. Microbiol. **32:**1079–1082.
21. **Tytgat, G. N. J., A. T. R. Axon, M. F. Dixon, D. Y. Graham, A. Lee, and B. J. Marshall.** 1990. World Congresses of Gastroenterology, Working Party Reports 36–45. Blackwell Scientific Publications, Melbourne, Australia.
22. **van Zwet, A. A., J. C. Thijs, J. A. J. Oom, J. Hoogeveen, and B. L. Düringshoff.** 1993. Failure to eradicate *Helicobacter pylori* in patients with metronidazole resistant strains. Eur. J. Gastroenterol. Hepatol. **5:**185–186.
23. **van Zwet, A. A., J. C. Thijs, W. Schievink-de Vries, J. Schiphuis, and J. A. M. Snijder.** 1994. In vitro studies on stability and development of metronidazole resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **38:**360–362.
24. **Wang, Y., K. P. Roos, and D. E. Taylor.** 1993. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J. Gen. Microbiol. **139:**2485–2493.