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In Vitro Selection of Resistant *Helicobacter pylori*

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Four strains of *Helicobacter pylori* were subjected to an in vitro serial passage technique to compare the propensity of the organisms to develop resistance to seven classes of antibacterial agents. The passages were made on serially doubling concentrations of antibacterial agents incorporated into agar starting at one-half the base-line MIC. The frequency of spontaneous resistance was also determined for each strain at four and eight times the MIC of each antibacterial agent. Strains resistant to ciprofloxacin, metronidazole, erythromycin, and tobramycin were isolated. The experiments failed to select organisms resistant to bismuth subsalicylate, furazolidone, or amoxicillin, although the MIC of amoxicillin was increased 4- to 16-fold. With the exception of erythromycin, organisms with the selected resistance were stable after at least three passages on antibacterial agent-free medium. Spontaneous resistance rates were generally of a low magnitude and were not predictive of the serial passage results.

Helicobacter pylori (formerly *Campylobacter pylori*) is accepted as the most common and most important cause of chronic active gastritis and represents one of the common chronic infections of humans. *H. pylori* infection and gastritis are also associated with the vast majority of cases of chronic, recurrent duodenal ulcer, with a growing body of data supporting an important etiologic role for the organism in that disease (4, 7).

Antibacterial therapy resulting in the clearance of *H. pylori* from the gastric mucosa has led to histologic improvement or normalization of chronic active gastritis (5, 17, 19). In patients with duodenal ulcers associated with *H. pylori* infections, effective eradication of the organism has resulted in improved healing of ulcers (12) and significantly lower rates of ulcer relapse (3, 12). These findings support a role for antibacterial therapy in the treatment of *H. pylori*-associated upper gastrointestinal diseases.

Two major problems have been encountered in the treatment of *H. pylori* infections, despite its in vitro susceptibility to a wide array of antibacterial agents. First, relapse of *H. pylori* is common following what appears to be successful treatment (5, 17, 19). Relapse is presumably due to incomplete eradication of the organism. The second major problem has been the emergence of resistant strains during single-agent treatment. The development of resistance has been reported during treatment with several quinolones (10, 16; Y. Glupczynski, M. Labbe, A. Burette, M. Delmee, V. Avesani, and C. Bruck, Letter, Lancet i:1096, 1987; J. W. Stone, R. Wise, I. A. Donovan, and J. Gearty, Letter, J. Antimicrob. Chemother. 22:92–93, 1988), nitroimidazoles (6, 10), and rifampin (10).

The purpose of this in vitro study was to compare the propensity of *H. pylori* strains to develop resistance to several antibacterial agents by using a serial passage technique (22) and to investigate the rates of spontaneous resistance to the study drugs.

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MATERIALS AND METHODS

Organisms. One commercially available strain of *H. pylori* (ATCC 43526) and three clinical isolates kindly provided by Mohamed Karmali (Hospital for Sick Children, Toronto, Ontario, Canada) were used for this study. The identity of *H. pylori* was confirmed by colony appearance, Gram stain, and positive biochemical tests (oxidase, catalase, and urease). After initial isolation, all strains were subcultured at least three times before they were studied. Throughout this report the following abbreviations are used to identify the strains: HP-2, ATCC 43526; HP-3, HSC 751311; HP-4, HSC 8008345; and HP-5, HSC 767506.

Antibacterial agents. Reference standards for the seven antimicrobial agents were provided by the manufacturers. The antimicrobial agents studied were amoxicillin (Beecham Laboratories, Bristol, Tenn.), bismuth subsalicylate (Davos Chemicals, Fort Lee, N.J.), ciprofloxacin (Miles Laboratories, Inc., West Haven, Conn.), erythromycin (Abbott Laboratories, North Chicago, Ill.), furazolidone (Norwich Eaton Pharmaceuticals, Norwich, N.Y.), metronidazole (G. D. Searle, Skokie, Ill.), and tobramycin (Eli Lilly & Co., Indianapolis, Ind.).

Determination of MICs. MICs were determined by a routine agar dilution technique (1) by using Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.25% yeast extract (Difco Laboratories, Detroit, Mich.) and 5% lysed sheep erythrocytes.

Stock solutions of tobramycin, metronidazole, and ciprofloxacin were prepared with sterile water. Amoxicillin stock solutions were prepared each day with a 0.1 M phosphate buffer solution (pH 6) (18). Erythromycin was initially dissolved in acetonitrile and was then further diluted with sterile water. Furazolidone solutions were prepared daily with *N*,*N*-dimethylformamide prior to further dilution with sterile water. At the concentrations used, neither acetonitrile nor dimethylformamide inhibited the growth of *H. pylori*. Bismuth subsalicylate was dispersed in a small volume of glycerol and then suspended in a 1% solution of microcrystalline cellulose (Avicel; FMC Corp., Philadelphia, Pa.). Serial dilutions of all of the study drugs were prepared with sterile water, except for amoxicillin, which was diluted with the phosphate buffer. All antibacterial agents were added to

TABLE 1. MICs at the base line and after serial passage experiments

Drug	MIC (μg/ml)							
	HP-2		HP-3		HP-4		HP-5	
	Base line	After passage	Base line	After passage	Base line	After passage	Base line	After passage
Amoxicillin	0.03	0.125	0.016	0.25	0.004	0.06	0.016	0.016[a]
Metronidazole	32	32[a]	1.0	64	1.0	—[b]	1.0	—
Furazolidone	0.25	0.25[a]	0.06	0.125	0.06	0.06[a]	0.125	0.125[a]
Bismuth subsalicylate	16	16[a]	4.0	4.0[a]	8.0	8.0[a]	8.0	8.0[a]
Ciprofloxacin	0.5	8.0	0.25	16	0.125	16	0.5	16
Erythromycin	0.5	0.5[a]	0.125	2.0	0.25	8.0	0.125	1.0
Tobramycin	0.25	4.0	0.5	—	0.25	0.25[a]	0.5	>16

[a] Serial passage experiment failed to isolate a more resistant strain.
[b] —, Serial passage strain was lost to overgrowth by a contaminant.

the media at 1:100 dilutions. Antibacterial agent-containing medium was prepared within 24 h of use and stored overnight at 4°C if necessary. Amoxicillin plates were always prepared on the day of use.

Isolates were grown for 72 h on blood agar and then suspended in tryptic soy broth to provide a turbidity approximating a 0.5 McFarland standard. A 1-μl inoculum was applied by using a multipoint inoculator (MIC-2000 Inoculator; Dynatech Laboratories, Inc., Alexandria, Va.). The inoculum ranged from 0.36×10^5 to 5.5×10^5 CFU. Isolates of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were included on each plate to serve as control organisms.

The plates were incubated for 72 h at 37°C in a humid, microaerophilic environment (CampyPouch System; BBL). The MIC was defined as the lowest concentration (micrograms per milliliter of agar) that inhibited visible growth, disregarding a haze of barely visible growth. MIC testing was performed in duplicate.

MIC interpretive standards for *H. pylori* have not been established. For the purposes of this study, the MIC was interpreted as a susceptible or resistant result according to the guidelines of the National Committee for Clinical Laboratory Standards for aerobic organisms where applicable (18).

Serial passage technique. After determination of the base-line MIC, each *H. pylori* strain was subjected to a serial passage experiment for each antibacterial agent as described by Tenney et al. (22), using the media and incubation conditions described above. A 72-h growth of each *H. pylori* strain was transferred with a swab onto agar containing one-half the MIC of each study drug. Surface growth after 72 h of incubation was transferred with a swab onto antibacterial agent-free medium for isolation and was also transferred to medium containing twice the prior concentration of drug. These plates were then incubated for 72 h. This process was repeated serially until no growth occurred or a predefined maximum antibacterial concentration was reached. The MIC of each of the compounds examined in this study was determined for isolates that were grown with the highest concentration of drug.

Isolates were also transferred at least three times on antibacterial agent-free medium, followed by a redetermination of the MIC to assess the stability of the selected resistance.

Frequency of spontaneous resistance. Duplicate plates containing the media and antibacterial agents described above were prepared at four and eight times the MIC for each original *H. pylori* isolate. A suspension (0.1 ml) of each isolate prepared from a 72-h culture was spread over the surface of the plate. The inoculum ranged from 7.2×10^5 to 2.9×10^7 CFU. The plates were incubated for 5 days. The frequency of spontaneous resistance was calculated by dividing the colony count on the antibacterial agent-containing plates by the inoculum.

RESULTS

Serial passage results. The MICs for the original *H. pylori* isolates are given in Table 1 and are labeled as base-line results. Based on the interpretive standards for aerobic organisms of the National Committee for Clinical Laboratory Standards, all strains were susceptible to amoxicillin, ciprofloxacin, erythromycin, and tobramycin. Although interpretive standards have not been established, all strains were susceptible to low concentrations of bismuth subsalicylate, furazolidone, and metronidazole, with the exception that strain HP-2 was not susceptible to metronidazole. When the *H. pylori* isolates were serially transferred on agar plates containing doubling concentrations of antibacterial agents, the MICs of several agents were increased 4- to 128-fold.

Resistant strains were selected with ciprofloxacin, tobramycin, metronidazole, and erythromycin. Although the amoxicillin MIC could be increased 4- to 16-fold, resistant isolates remained susceptible to 0.25 μg or less of the drug per ml. The serial passage technique was unsuccessful at selecting isolates that were less susceptible to bismuth subsalicylate or furazolidone.

Cross resistance was an unusual occurrence. The resistant HP-5 isolate selected on ciprofloxacin demonstrated a 32-fold increase in the metronidazole MIC, to 32 μg/ml. Following serial passage on plates with increasing furazolidone concentrations, the HP-2 isolate that was recovered had a fourfold increase in the tobramycin MIC, to 1.0 μg/ml, even though there was no change in its susceptibility to furazolidone. The HP-3 strain that was isolated during the furazolidone serial passage had an eightfold increase in the metronidazole MIC, to 8.0 μg/ml, while its susceptibility to furazolidone showed a minimal change.

After three or more transfers on antimicrobial agent-free medium, the MICs for strains that were isolated during passage on erythromycin decreased two- to fourfold. For resistant strains that were isolated with ciprofloxacin, metronidazole, tobramycin, and amoxicillin, the MICs remained stable. The combined resistance reported for the two furazolidone isolates (HP-2 and HP-3) was stable.

Spontaneous resistance. The frequencies of the spontaneous emergence of resistance in the isolates to four and eight times the MICs of the antimicrobial agents are given in Table 2. The highest rates of spontaneous resistance were ob-

TABLE 2. Frequency of spontaneous resistance at four or eight times the MIC

Drug	Frequency of spontaneous resistance[a]							
	HP-2		HP-3		HP-4		HP-5	
	4× MIC	8× MIC	4× MIC	8× MIC	4× MIC	8× MIC	4× MIC	8× MIC
Amoxicillin	1.8×10^{-7}	$<5.2 \times 10^{-8}$	$<1.4 \times 10^{-6}$	$<1.4 \times 10^{-6}$	$<3.5 \times 10^{-8}$	$<3.5 \times 10^{-8}$	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$
Metronidazole	5.0×10^{-5}	2.6×10^{-5}	6.9×10^{-7}	$<1.4 \times 10^{-6}$	—[b]	$<3.5 \times 10^{-8}$	8.7×10^{-6}	5.7×10^{-6}
Furazolidone	$<5.2 \times 10^{-8}$	$<5.2 \times 10^{-8}$	6.9×10^{-7}	$<1.4 \times 10^{-6}$	—	—	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$
Bismuth subsalicylate	$<1.6 \times 10^{-7}$	$<1.6 \times 10^{-7}$	$<1.4 \times 10^{-6}$	$<1.4 \times 10^{-6}$	7.0×10^{-8}	$<3.5 \times 10^{-8}$	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$
Ciprofloxacin	$<1.6 \times 10^{-7}$	$<1.6 \times 10^{-7}$	$<1.4 \times 10^{-6}$	$<1.4 \times 10^{-6}$	1.7×10^{-8}	$<3.5 \times 10^{-8}$	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$
Erythromycin	4.0×10^{-7}	3.2×10^{-7}	6.9×10^{-7}	1.9×10^{-5}	$<3.5 \times 10^{-8}$	$<3.5 \times 10^{-8}$	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$
Tobramycin	4.3×10^{-5}	4.2×10^{-9}	1.4×10^{-6}	$<1.4 \times 10^{-6}$	5.2×10^{-8}	$<3.5 \times 10^{-8}$	$<6.0 \times 10^{-8}$	$<6.0 \times 10^{-8}$

[a] Values with a less than symbol indicate that no mutants were detected.
[b] —, Plates were contaminated.

served with strain HP-2 and metronidazole and tobramycin and with strain HP-5 and metronidazole. The level of detection for these experiments was limited by the size of the inoculum; this was especially true for HP-3.

DISCUSSION

H. pylori is highly susceptible in vitro to many classes of antibacterial agents and bismuth salts (9, 11, 13, 21). Our MIC results for the four original *H. pylori* isolates are in agreement with those results that have been previously reported.

Despite the susceptibilities reported with in vitro testing systems, it has been very difficult to achieve long-term eradication of *H. pylori* infections, and a reliable and safe regimen has not been discovered (7). The most common microbiologic outcomes following antibacterial therapy have been initial clearance of the organism followed by relapse or recurrent infection within 6 weeks (5, 17, 19), failure to eradicate the organism which remains susceptible to the regimen (5, 10, 17), or the emergence of a resistant *H. pylori* infection during treatment (6, 10, 16; Glupczynski et al., Letter, Lancet i:1096, 1987; Stone et al., Letter, J. Antimicrob. Chemother. 22:92–93, 1988).

The development of resistance to antibacterial agents has been most frequently described during trials involving fluoroquinolones. In a placebo-controlled trial using ofloxacin, Glupczynski et al. (Glupczynski et al., Letter, Lancet i:1096, 1987) reported a rise in the ofloxacin MIC from 0.25 to 0.5 μg/ml before therapy to 16 to 32 μg/ml after therapy. There was no change in the MIC for isolates from the patients who received a placebo. Mertens and co-workers (16) showed a similar emergence of resistance during treatment of *H. pylori* with norfloxacin. MIC results for 11 patients who had susceptible isolates prior to treatment were available for these patients after they were treated with norfloxacin, and isolates from 9 of these patients showed newly acquired resistance. Treatment failure in this trial was nearly universal. Acquired resistance and failure to eradicate *H. pylori* has also been described with ciprofloxacin (10; Stone et al., Letter, J. Antimicrob. Chemother. 22:92–93, 1988; M. Sachdeva, B. L. Lee, and M. A. Sande, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 41, 1989).

Results of our in vitro studies demonstrated parallel findings with exposure of *H. pylori* to subinhibitory concentrations of ciprofloxacin. Selection of ciprofloxacin resistance occurred easily with all four isolates tested, and this resistance was stable over the time period studied. The magnitude of the MICs observed before and after treatment in the clinical studies reporting ciprofloxacin resistance were similar to the MICs found in this study.

McNulty and co-workers (14) measured the concentrations of several drugs in gastric biopsy specimens following the administration of usual doses. Following the administration of ciprofloxacin at 500 mg, high concentrations were achieved for up to 6 h after the dose; however, after 8 h the drug was undetectable. If ciprofloxacin is administered on the usual 12-h schedule, there may be a prolonged interval of subinhibitory concentrations at the site of infection which may be conducive to the selection of resistant strains.

Selection of resistant *H. pylori* isolates has been described during therapy with the nitroimidazoles tinidazole and metronidazole. Goodwin et al. (6) enrolled 100 patients in a double-blind placebo-controlled trial in which they compared four treatment arms. In the group that received cimetidine and tinidazole, 19 of 27 patients (70%) with susceptible *H. pylori* isolates before treatment had resistant isolates following treatment. In the group that received combination therapy with tinidazole and a bismuth salt, emergence of resistance still occurred but at a significantly lower rate, with just 2 of 22 initially susceptible isolates becoming resistant. Hirschl et al. (10) reported the development of nitroimidazole resistance in isolates obtained from two of four patients treated with metronidazole.

The serial passage technique used in the present study was able to isolate a metronidazole-resistant strain of *H. pylori*. Two other strains also grew on increased concentrations of metronidazole, but were lost prior to final isolation. As with ciprofloxacin, our in vitro results with a nitroimidazole parallel what has been shown to occur in vivo. The magnitude of the MIC achieved for the resistant isolate was similar to the MICs reported for resistant strains recovered during in vivo studies (6, 10).

Although MIC interpretive standards have not been described for metronidazole, maximal concentrations in serum with usual therapeutic doses are approximately 25 μg/ml (20). Therefore, isolates for which MICs exceed achievable concentrations in serum could reasonably be considered resistant.

Emergence of resistance during erythromycin therapy has not been reported. In the one clinical trial with erythromycin monotherapy, *H. pylori* was cleared from only 1 of 15 patients. Pre- and posttreatment in vitro susceptibility data for the isolates were not reported. The investigators theorized that therapeutic failure was a result of the use of an inactive erythromycin ester coupled with an inability to

achieve bactericidal concentrations of the free erythromycin base at the site of infection (15).

Azythromycin, a macrolide similar to erythromycin, has been unsuccessfully used as monotherapy for *H. pylori* (Y. Glupczynski and A. Burette, Letter, Am. J. Gastroenterol. **85**:98, 1990). Susceptibility results were available for the pre- and posttherapy isolates obtained from six of the patients. In four patients, pretherapy strains were susceptible (MIC, 0.25 μg/ml), while the posttherapy strains were resistant to azythromycin (MIC, >64 μg/ml). In the other six patients without pretherapy isolates, the posttherapy isolates were similarly resistant to azythromycin. All of the resistant strains demonstrated cross resistance to erythromycin.

In vitro, we were able to increase the erythromycin MIC for three *H. pylori* strains above the normally accepted susceptible range, and the MIC for one strain was achieved in the resistance range (18). If similar selection of resistant strains occurs in vivo, an additional possible explanation for the observed therapeutic failure could be the emergence of erythromycin resistance similar to that reported with azythromycin.

Aminoglycoside antibiotics have not been used clinically in the treatment of *H. pylori* infections, presumably because of an unacceptable risk of toxicity with systemic therapy. We included tobramycin in our study to represent an additional class of agents with in vitro activity against *H. pylori*. The ability to select strains in vitro which are less susceptible to tobramycin may provide an additional reason to exclude systemic aminoglycosides from clinical trials of treatments for *H. pylori* infections.

Bismuth subsalicylate, furazolidone, and amoxicillin serial passage experiments did not result in the selection of resistant strains. Bismuth salts have been used in a large number of studies of *H. pylori*, but pre- and posttreatment susceptibility data are available from only one. Goodwin et al. (C. S. Goodwin, B. Bell, C. McCullough, and M. Turner, Letter, J. Clin. Pathol. **42**:216–217, 1989) reported unchanged MIC results before and after treatment with colloidal bismuth subcitrate for 15 isolates obtained from patients who were unsuccessfully treated. They also attempted to select resistant organisms in vitro, but were unsuccessful. When combined with our data, these findings suggest that the emergence of bismuth resistance is unlikely to be an important cause of treatment failure.

Furazolidone is a nitrofuran with considerable activity against *H. pylori* in vitro (13). Published experience with the use of furazolidone in the treatment of *H. pylori* is limited. Morgan et al. (17) included a furazolidone oral suspension in a double-blind clinical trial. Of the 14 patients treated, 13 achieved initial clearance of *H. pylori*; however, at 6 weeks 58% of the evaluable patients had relapses. All recovered isolates were susceptible to furazolidone, indicating that none of the relapsing infections was caused by resistant organisms. Graham and co-workers (8) have reported on the only other *H. pylori* trial involving furazolidone. That trial reported in vivo susceptibility as assessed by urea breath testing for the presence of *H. pylori*; therefore, no in vitro susceptibility data were available from that study. When combined with the results reported by Morgan et al. (17), our data suggest that treatment failure secondary to the emergence of furazolidone resistance would be unlikely. This is especially true since furazolidone is not appreciably absorbed and reaches very high intraluminal concentrations with usual doses (2). The MIC for an organism that was truly resistant to furazolidone would be very high.

Amoxicillin treatment of *H. pylori* infections has not been reported to result in the emergence of resistant strains; however, only one trial commented on the susceptibility of the isolates. Glupczynski et al. (5) stated that no amoxicillin resistance was detected in isolates from patients who had relapses after treatment. Hirschl and co-workers (10) reported that for patients treated with bacampicillin, the pre- and posttreatment MIC of ampicillin was unchanged, despite an 80% treatment failure rate.

In the present study, the serial passage procedure was able to select strains for which the MIC of amoxicillin was higher than that for the original isolate, but the organisms were still susceptible to amoxicillin. This suggests that selection of relatively more resistant strains is not of clinical importance since they remain susceptible to normally achievable concentrations, unless the concentration at the site of the *H. pylori* infection is considerably lower than anticipated.

The spontaneous resistance rates were generally low and did not correlate well with the ability to select resistant strains on serial passage, with the possible exception of metronidazole. For example, spontaneous resistance to ciprofloxacin was essentially below the level of detection, yet the selection of resistance to ciprofloxacin was easily achieved with all four isolates. Spontaneous resistance rates previously reported for one strain of *H. pylori* were of a similarly low magnitude (9).

The mechanisms of resistance for *H. pylori* have not been reported previously and were not the subject of this study. It is possible that several mechanisms are involved in conferring the resistance observed in this study, and these may or may not be relevant in vivo.

In conclusion, *H. pylori* strains that were resistant to ciprofloxacin, metronidazole, erythromycin, and tobramycin could be selected in vitro. Isolates resistant to amoxicillin, furazolidone, and bismuth subsalicylate could not be selected. Limited data from clinical trials show similar patterns of resistance selection in vivo. This correlation between in vitro and in vivo outcomes suggests that knowledge of the frequency and level of resistance that can be selected by the serial passage technique may be an important consideration when selecting drugs in the design of clinical trials or therapeutic regimens.

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