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Bactericidal Activity of Antimicrobial Agents against Slowly Growing *Helicobacter pylori*

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The doubling times of bacteria at sites of colonization or infection are considerably longer than those in laboratory culture media, and slow growth reduces the susceptibility of bacteria to antimicrobial agents. *Helicobactor pylori* is susceptible to a wide range of antimicrobial agents in vitro; however, tests for inhibitory activity do not adequately predict which antimicrobial agents will eradicate slowly growing *H. pylori* from the stomachs of patients. The chemostat can be used to compare the bactericidal activities of antimicrobial substances against slowly growing bacteria. In this study we compared the bactericidal activities of antimicrobial agents against slowly growing *H. pylori*. The bactericidal activities of erythromycin, minocycline, ampicillin, amoxicillin, cefixime, metronidazole, and bismuth subcitrate against slowly growing *H. pylori* NCTC 11,637 in a chemostat were compared. Antimicrobial agents were added to the system at four to eight times the MIC. Exposure of *H. pylori* to metronidazole was associated with the rapid development of metronidazole resistance, preventing assessment of the bactericidal activity of metronidazole. Resistance to the other antimicrobial agents tested did not develop. The poor bactericidal activities of the antimicrobial agents against slowly growing *H. pylori* may be a contributory factor in limiting their clinical efficacies. Of the agents tested, only amoxicillin and bismuth subcitrate showed bactericidal activity against slowly growing *H. pylori*. The chemostat allows comparison of the bactericidal activities of antimicrobial agents against slowly growing *H. pylori* and may therefore provide results which more accurately identify those agents or combinations of agents that will eradicate *H. pylori* from patients.

Helicobacter pylori probably contributes to the pathogenesis of antral gastritis and peptic ulcer disease (3). Eradication of *H. pylori* is associated with a reduction in the relapse rate of peptic ulcer disease (15).

H. pylori is susceptible to a wide range of antimicrobial agents in vitro, but eradication usually requires the use of combinations of agents (3, 4, 8) such as amoxicillin and bismuth subcitrate. Bacteria grow slowly at sites of infection (7, 13). The bactericidal activities of antimicrobial agents against slowly growing bacteria may be greatly reduced because of changes in outer membrane permeability, peptidoglycan structure, or penicillin-binding proteins (6). The influence of growth rate on the susceptibilities of members of the family *Enterobacteriaceae* (6) and *Pseudomonas aeruginosa* (1) to antimicrobial agents has been studied in a chemostat model. We describe here the use of a chemostat to study the susceptibilities of slowly growing *H. pylori* to antimicrobial agents.

MATERIALS AND METHODS

A stable continuous culture of *H. pylori* NCTC 11,637 was established in an LH 500 direct-drive fermentor (LH Fermentation; Stoke Poges, Slough, United Kingdom). The growth medium was a modified brucella broth containing 0.5% fetal calf serum and sodium pyruvate (0.25 g/liter). A gas mixture consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen was bubbled through the chemostat vessel at a rate of 300 ml/min. The stir rate was maintained at 700 rpm. The pot volume at this stir rate was 700 ml. Broth was added to the chemostat vessel at a rate of 29 ml/h (700 ml/24 h). The viable count usually stabilized 7 to 10 days after inoculation of the vessel. When the viable count was stable, the oxygen concentration remained in the range of 4 to 6%. The pH was maintained at 7.0 by using 2 M sodium hydroxide or 2 M hydrochloric acid. This pH was chosen because it probably represents most closely that of the pH at the apices of gastric epithelial cells (16).

Bismuth subcitrate was provided by Gist-Brocades (Delft, Holland). Chloramphenicol and metronidazole were obtained from Sigma Chemical Co. (Poole, United Kingdom). Erythromycin was obtained from Abbott (Queenborough, United Kingdom). Cefixime and minocycline were obtained from Lederle Ltd. (Gosport, United Kingdom), and ampicillin and amoxicillin were from Beecham Laboratories (Betchworth, United Kingdom). The MICs of the antimicrobial agents were determined by a plate inclusion method with heated blood agar, and repeat determinations were done by a broth dilution method (with modified brucella broth) (12). The results obtained by both methods were similar to those obtained for NCTC 11,637 by Goodwin et al. (9), using a broth dilution method. Antimicrobial agents were added directly to the broth within the vessel through an injection port to give a final concentration of four to eight times the MIC at the start of the experiment and to ensure that supra-MICs were present in the chemostat for 24 h.

A sample of 0.5 ml was removed from the chemostat vessel for viable count determinations. The sample was serially diluted 10-fold in Tryptone soy broth (Oxoid CM 129B), and 10- and 100-μl volumes from each dilution were spread onto heated blood agar plates. Inoculated plates were incubated under microaerophilic conditions at 37°C for 7 days before colonies were counted. Samples were collected for viable count determinations immediately preceding addition of the antimicrobial agents to the chemostat vessel and at 2, 4, 6 (or 8), and 24 h after addition of the antimicrobial

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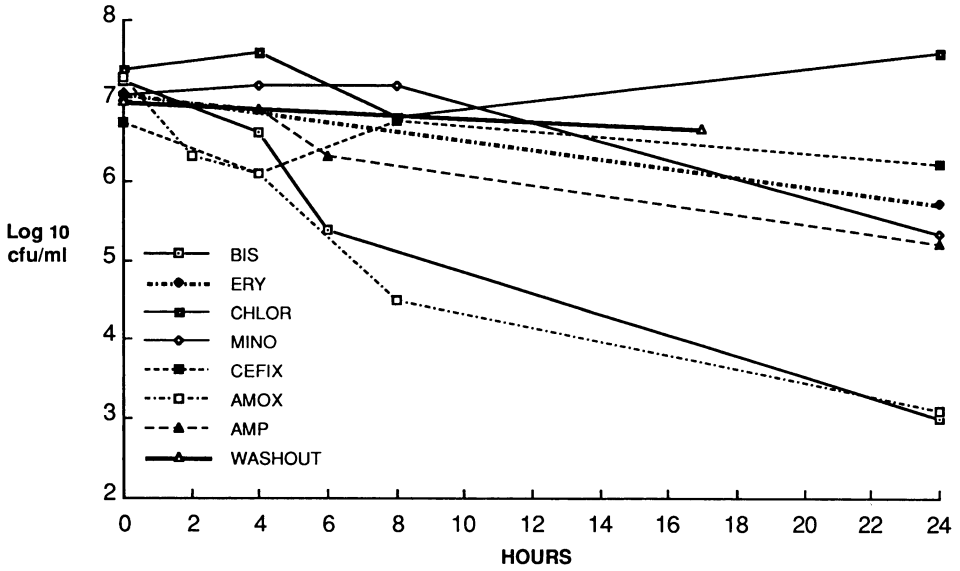

FIG. 1. Bactericidal activities of antibiotics. BIS, bismuth subcitrate; ERY, erythromycin; CHLOR, chloramphenicol; MINO, minocycline; CEFIX, cefixime; AMOX, amoxicillin; AMP, ampicillin.

agents. The antimicrobial susceptibility of *H. pylori* NCTC 11,637 24 h after exposure to the antimicrobial agents was compared with that of NCTC 11,637 from a heated blood agar plate subculture (without antimicrobial agents) by disk diffusion testing. The presence of antimicrobial activity in the chemostat broth 24 h after addition of the antimicrobial agents was confirmed by a microbiological plate assay (12) with *H. pylori* NCTC 11,637.

Each experiment was repeated at least once to ensure reproducibility.

RESULTS

The MICs of antimicrobial agents for *Helicobacter pylori* NCTC 11,637 in modified brucella broth were 0.25 mg/liter for erythromycin, 0.25 mg/liter for minocycline, 0.12 mg/liter for ampicillin, 0.25 mg/liter for amoxicillin, 0.5 mg/liter for cefixime, 4 mg/liter for chloramphenicol, 0.5 mg/liter for metronidazole, and 8 mg/liter for bismuth subcitrate.

The viable counts of slowly growing *H. pylori* NCTC 11,637 following addition of the antimicrobial agents are shown in Fig. 1. Addition of metronidazole did not result in a reduction of the viable count by 8 or 24 h. At 24 h, the MIC of metronidazole for bacteria from the chemostat increased to a level similar to the concentration in the vessel at the start of the experiment. Resistance to the other antimicrobial agents tested did not develop. Only the addition of amoxicillin or bismuth subcitrate was associated with bactericidal activity (less than 0.1% survival at 24 h). Inhibitory activity was present in the chemostat broth 24 h after the addition of all of the antimicrobial substances tested.

DISCUSSION

Poor penetration into gastric mucus and inactivation by low pH may be factors that contribute to the limited clinical efficacies of antimicrobial agents that are active in vitro against *H. pylori*. Slow growth reduces the bactericidal activities of antimicrobial agents against *Escherichia coli* (6) and *P. aeruginosa* (1). In this study the bactericidal activities of antimicrobial agents against slowly growing *H. pylori* were studied, because it may be that antimicrobial agents that are bactericidal against slowly growing *H. pylori* are more likely than those that are bacteriostatic to eradicate *H. pylori* from patients.

Bismuth salts and amoxicillin are both commonly used in antimicrobial regimens for the eradication of *H. pylori* (3, 4, 8), and both of these antimicrobial agents showed bactericidal activity against slowly growing *H. pylori*. Previous studies have shown that amoxicillin is more bactericidal than ampicillin against *E. coli* in experimental infections in mice (11) and against slowly growing *E. coli* 205 in a chemostat (6). The results of this study suggest that amoxicillin is more bactericidal than ampicillin against slowly growing *H. pylori*. Armstrong et al. (2) demonstrated the bactericidal activity of bismuth salts against *H. pylori* in liquid media, although the bactericidal activity was less rapid than that in this study.

Metronidazole resistance is not uncommon in strains of *H. pylori* from patients previously treated with metronidazole (8, 10), and development of resistance prevented the assessment of the bactericidal activity of metronidazole. The lack of inhibitory activity of metronidazole was unexpected and suggests a change in the phenotype of the population rather than selection of a resistant subpopulation. None of the other antimicrobial agents tested have been shown to be effective at eradicating *H. pylori* from the stomachs of patients, and none showed bactericidal activity against slowly growing *H. pylori*. Otto et al. (14) were unsuccessful in attempts to eradicate *Helicobacter mustelae* from the stomachs of ferrets using orally administered chloramphenicol, and in the present study chloramphenicol was not bactericidal against *H. pylori*. Cefixime has been shown to be bactericidal against gram-negative bacterial species such as *E. coli* (5). The rate of reduction in viable count following the addition of cefixime to the chemostat approximated that expected from the washout of nongrowing cells, suggesting a bacteriostatic activity against slowly growing *H. pylori*.

Eradication of *H. pylori* is associated with a reduction in the rate of relapse of peptic ulcer disease. Current strategies for the eradication of *H. pylori* make use of combinations of antimicrobial agents, but even the most effective regimens

fail to eradicate *H. pylori* from 20% of patients. There is a need for improved antimicrobial regimens for the eradication of *H. pylori*. The chemostat provides a method for comparing the bactericidal activities of antimicrobial agents against slowly growing *H. pylori* and may therefore provide results which more accurately identify those agents or combinations of agents that will eradicate *H. pylori* from patients.

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