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Utilization of Time-Kill Kinetic Methodologies for Assessing the Bactericidal Activities of Ampicillin and Bismuth, Alone and in Combination, against *Helicobacter pylori* in Stationary and Logarithmic Growth Phases

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Assessment of in vitro susceptibility testing of *Helicobacter pylori* is difficult because of the fastidious, slowly growing nature of this microorganism. The high rate of relapse observed clinically and a possible subpopulation of cells that are not actively replicating suggest the potential need for bactericidal therapy in order to eradicate *H. pylori*. We used modified time-kill kinetic methodology in order to evaluate the bactericidal activities of ampicillin and bismuth, alone and in combination, against three strains of *H. pylori* in both a stationary (slow) growth phase and a logarithmic (rapid) growth phase. We found that ampicillin produced a decrease in CFU per milliliter (2 to 4 $\log_{10}$ units) for three strains of *H. pylori* when tested in logarithmic growth phases but was less inhibitory (<1-$\log_{10}$-unit decrease in CFU per milliliter) when tested in a stationary growth phase. In contrast, bismuth, when tested in a logarithmic growth phase, produced little inhibitory effect, as the CFU for all strains tested increased above the inoculum. However, when tested in a stationary growth phase, bismuth produced a decrease in CFU per milliliter of <1 to >3 $\log_{10}$ units). The activities of these two agents when combined mimicked the activity of the most active drug alone for that growth phase. We conclude that the clinical use of ampicillin combined with bismuth has been more effective than that of either agent used alone because ampicillin targets replicating cells, whereas bismuth targets cells that are not actively replicating.

Infection of gastrointestinal mucosal tissues with *Helicobacter pylori* has been causally associated with gastric and duodenal ulcers (2, 4, 16). In patients with *H. pylori* mucosal infections, eradication of this microorganism seems to be curative of both infection and ulcer disease (17, 25, 34). Antimicrobial therapy of *H. pylori* infections empirically has included single, double, and triple antibiotic combinations. To date, therapy with multiple antimicrobial agents appears to achieve the best clinical outcomes, but the optimal therapy of this infection has yet to be determined (13).

Unfortunately, the fastidious, slowly growing nature of *H. pylori* does not readily lend itself to conventional susceptibility test methods (10). Although modifications of standardized methods have been utilized, these are often technically difficult to perform and frequently exhibit poor reproducibility. Moreover, these methods are controversial because the results do not always correlate with the clinical outcome (12).

Clinical observations with empiric therapy with single agents against *H. pylori* have suggested a need for prolonged therapy, yet a high rate of relapse continues to exist despite such prolonged therapy. Double- or triple-antimicrobial-agent therapy has been found to be most effective and allows a shorter course of therapy (23). Relapse continues to be a problem (11). These observations raise important questions about the levels of antimicrobial agents in infected mucosal tissues, the possibility of populations of *H. pylori* that are heterogenetic in terms of the expression of resistance, the role of the metabolic status of *H. pylori* in infected mucosal tissues, and the potential necessity for bactericidal therapy.

Because the chronic nature of *H. pylori* infection implies a subpopulation of microorganisms that are metabolically inactive, and because relapse suggests the need for bactericidal activity, we investigated the bactericidal activities of ampicillin and bismuth, alone and in combination, against strains of *H. pylori*. Both agents are commonly used in the therapy of *H. pylori* infection, yet neither agent is as clinically effective when used alone as it is when used in combination. We evaluated the role of the metabolic status of *H. pylori* in response to these antimicrobial agents by using methodology which allowed bactericidal activity to be tested in a stationary (slow) growth phase as well as a logarithmic (rapid) growth phase.

(Some of these data were presented previously [8].)

MATERIALS AND METHODS

Microorganisms. MICs and time-kill studies were performed with *H. pylori* ATCC 43504 and two clinical strains, Hp107 and Hp36. In both studies, inocula were prepared in a laminar flow hood in the following manner. Cation-adjusted Mueller-Hinton broth (18 ml; Difco Laboratories, Detroit, Mich.) and fetal calf serum (2 ml; Sigma Chemical Co., St. Louis, Mo.) were aseptically added to 50-ml sterile bottles (Becton Dickinson and Co., Sparks, Md.). Sterile rubber stoppers (The West Co., Phoenixville, Pa.) were then inserted, with aluminum seals hand crimped tightly to prevent air leakage. The medium was inoculated with a tuberculin syringe, and the bottle was flushed with a mixture of three gases (10% CO_2, 5% O_2, and 85% N_2) and shaken at 150 rpm at 37°C. Cells were passed twice overnight in order to ensure logarithmic-phase growth. On the second day, cells were viewed under a phase microscope. This was done for quality control purposes because cells in logarithmic-phase growth will typically show curved morphology without clumping or coccoid forms and will be actively motile.

MICs. MICs were performed by a modification of standard agar dilution

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procedures (29). Media contained Mueller-Hinton agar, 0.5% starch, and either ampicillin (Sigma) or colloidal bismuth subcitrate (Brocades Pharma bv, Delft, The Netherlands). Cell suspensions were prepared as described above, and 10^5 CFU were inoculated per spot with a replicating device at appropriate times. MICs were read after 3 to 5 days of incubation at 37°C under microaerophilic conditions (Oxoid Gas Generating Kit; Unipath Ltd., Hampshire, England). Because the bismuth MIC endpoints were often difficult to interpret, these results were checked by urease testing, i.e., cells scraped from the plate that was one dilution lower than the so-called MIC plate yielded a positive urease reaction. All agar dilution tests were performed in duplicate.

Time-kill studies. Time-kill kinetic studies were performed, with cells tested in both logarithmic and stationary growth phases. All kinetic studies were performed in cation-adjusted Mueller-Hinton broth containing 0.5% starch. Ampicillin and bismuth were tested alone and in combination at concentrations of 0.25 and 8 μg/ml, respectively. Logarithmic-phase growth was established as initially described for the inocula. For stationary growth phase testing, bottles that were not flushed (ambient atmosphere) and were shaken at 21°C instead of 37°C were found to be the most consistent method for approximation of stationary-phase growth, as demonstrated by the identity of the final CFU count at 24 h with that at 0 h. Bottles for both growth phases were inoculated at the same time with the same cell suspension to achieve a final concentration of 2×10^6 CFU/ml. At 0, 3, 7, and 24 h, samples were removed for colony counts. Undiluted samples of 200 and 500 μl were plated directly onto agar media. In addition, samples were serially diluted 10-fold, with subsequent colony counts determined by plating 20-μl amounts of each dilution onto campylobacter base agar (Difco) supplemented with 10% whole sheep blood, 10% horse serum, 0.1% cholesterol, 75 mg of Ca^{2+} per liter, 36 mg of Mg^{2+} per liter, and 3 μg of amphotericin B per ml (9). After inoculation, all spotted media were immediately transferred to a holding incubator with 10% CO_2 and high humidity; when a jar was filled, it was immediately closed and placed in the incubator. Samples were spotted onto duplicate plates. The lowest level of accurate cell detection by this method was 60 CFU/ml. After 4 to 6 days of incubation under microaerophilic conditions at 37°C, plates were read with a magnifying lens.

To eliminate antimicrobial carryover in the time-kill studies, penicillinase (1:1,000 dilution of Bacto Penase; Difco) or EDTA (5 mM) was added to the sample aliquots containing ampicillin or bismuth, respectively. Carryover was monitored in two ways. First, 200 μl of undiluted sample was streaked across the central portion of an agar plate. After the sample was allowed to be absorbed, the central portion was cross-streaked and the plate was incubated. Inhibition of growth in the central area in contrast to growth at the peripheral area was considered an indication of drug carryover (31). Carryover was also monitored by comparison of colony counts of samples taken from bottles with and without antimicrobial agents immediately after inoculation. Colony counts that differed by less than 5% were considered to have no antimicrobial carryover (35).

RESULTS

The ampicillin and bismuth MICs, respectively, for the three strains of *H. pylori* as determined by the agar dilution methodology were as follows: ATCC 43504, 0.06 and 32 μg/ml; Hp107, 0.25 and 64 μg/ml; and Hp36, 0.06 and 32 μg/ml. As frequently seen with agar dilution results for *H. pylori*, some endpoints were difficult to read.

The antimicrobial effects of ampicillin and bismuth against the three study strains when tested in a logarithmic growth phase are summarized in Fig. 1. Ampicillin at 0.25 μg/ml produced a decrease in CFU per milliliter for all three strains, achieving over a 24-h period a greater than 3-$\log_{10}$-unit decrease for two strains and a 2-$\log_{10}$-unit decrease for the third strain. In contrast, bismuth had no bactericidal activity against any of these strains. Moreover, the inhibitory effect of bismuth was minimal, with all strains exhibiting an increase in growth over the 24 h tested.

When tested in a stationary growth phase, the bactericidal effects of ampicillin and bismuth were essentially reversed. Ampicillin at 0.25 μg/ml was not bactericidal against any of the three strains tested, although inhibition of all strains was noted (Fig. 2). Bismuth, however, exhibited more activity in stationary growth phase at a concentration of 8 μg/ml, achieving a greater then 3-$\log_{10}$-unit decrease in CFU per milliliter for ATCC 43504 and a 2.7-$\log_{10}$-unit decrease for Hp107 over 24 h. Bismuth was only slightly inhibitory against Hp36, achieving less than a 1-$\log_{10}$-unit decrease in CFU per milliliter.

When tested in combination at the same concentrations and the same two growth phases, ampicillin and bismuth together

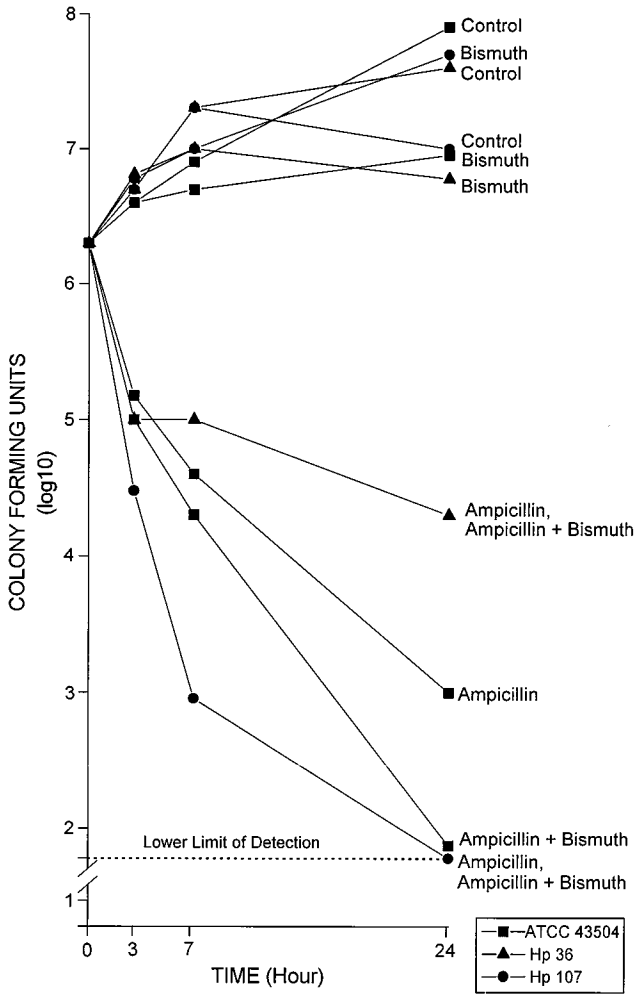

FIG. 1. Bactericidal activity of ampicillin (0.25 μg/ml) and bismuth (8 μg/ml) alone and in combination against three strains of *H. pylori* when tested in the logarithmic phase of growth.

mimicked the activity of the most active drug alone for the respective growth phase tested, although some enhancement of activity was observed for ampicillin plus bismuth against Hp36 in the stationary growth phase. No antibiotic carryover was observed for either ampicillin or bismuth in any of the time-kill kinetic studies performed.

DISCUSSION

All patients with *H. pylori* infection of the gastrointestinal mucosal surface have some form of gastritis, such as asymptomatic gastritis or peptic ulcer (2, 19). The association of *H. pylori* with such gastritis is the major reason that this pathogen is now thought to cause peptic ulcer disease (2, 19). *H. pylori* is found in 95% of patients with duodenal ulcers, in 70% of patients with gastric ulcers, and in some cases of chronic gastritis (2, 38). Moreover, treatment of the infection with long-term eradication of *H. pylori* is associated both with clearing of the gastritis and with healing of the ulcer (34).

Eradication of *H. pylori*, however, has proven very difficult. A number of antibiotics have been used alone, but such monotherapy has resulted in poor clinical results (12) despite in vitro

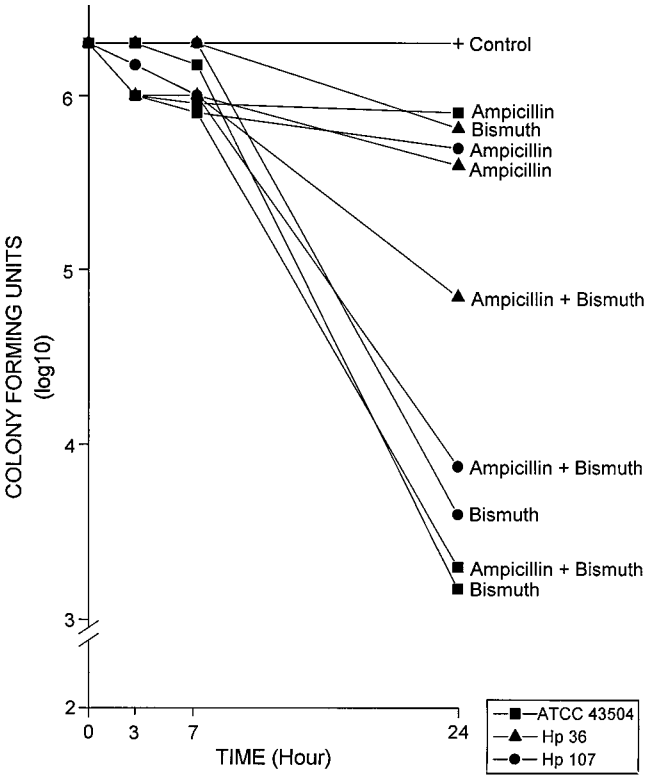

FIG. 2. Bactericidal activity of ampicillin (0.25 μg/ml) and bismuth (8 μg/ml) alone and in combination against three strains of *H. pylori* when tested in stationary-phase growth. The line marked control (+) is for all three strains.

susceptibility test results that suggest that the microorganism is susceptible to the single agents used (15, 21, 26).

The poor clinical results in these studies usually reflect a high rate of relapse after an initial period in which the eradication of *H. pylori* for many patients seems complete. These relapses have been shown to involve the original infecting strain (22, 37) as opposed to reinfection with different strains of *H. pylori*. In contrast, when triple-drug therapy has been used, the rate of relapse (or reinfection) has been low for many years after therapy (11).

Ampicillin is among the most active agents as determined by in vitro susceptibility testing (the MIC at which 90% of the isolates are inhibited is 0.01 μg/ml) (27, 28) and has been extensively studied for the therapy of *H. pylori* infections (3, 5, 14). In fact, of the single agents studied to date for therapy of *H. pylori* infection, amoxicillin, a derivative of ampicillin, has demonstrated the best efficacy (3). Amoxicillin results in the short-term eradication of this microorganism in 70 to 90% of patients but has a high rate of relapse, with only 20% of patients demonstrating long-term eradication after treatment (3, 5, 14).

Bismuth also has been extensively studied and has been shown to eliminate *H. pylori* in as many as 70% of treated patients (24). However, within 1 month most of these patients (90%) relapse, with recurrence of the infection as well as the associated gastritis or ulcer disease. The current thinking is that bismuth only transiently disrupts the *H. pylori* in gastric mucosa (2, 18). When bismuth is added to amoxicillin, the initial eradication rates approach 95% at the end of 4 weeks of therapy, but within 1 month after discontinuation of therapy almost 50% of these patients will again be culture positive for *H. pylori* (30, 33).

Poor penetration into gastric mucosa and inactivation by low pH are possible factors that may contribute to the limited clinical efficacy of ampicillin and bismuth (3). However, investigators in Germany have reported that the concentration of amoxicillin in the antra of six volunteers ranged from 0.04 to 0.42 μg/ml of ground biopsy tissue (7). This, along with an initial eradication rate of 70 to 90%, argues that inadequate gastric mucosal levels are not due either to poor penetration or to significant drug inactivation by gastric acid. In addition, the pharmacokinetics and pharmacodynamics of bismuth have been examined (1, 6, 20, 24, 36). The concentration of bismuth in human antral gastric mucosa ranged from a peak value of 375 μg/g at 1 to about 10 μg/g at 4 h after ingestion of a single dose of bismuth (20). Moreover, the fact that this agent can rapidly clear *H. pylori* from gastric mucus and epithelial surface membranes, as has been shown in sequential endoscopic biopsy studies (1), suggests that neither poor penetration nor drug inactivation is an important factor for the relapses observed when bismuth has been used as a single agent.

Another potential reason for a high rate of relapse is the development of resistance during initial therapy (12). Although this has been shown for *H. pylori* and metronidazole (32), development of resistance against ampicillin or bismuth has not been documented. Thus, resistance is unlikely to explain the high rate of relapse seen after initial eradication in most patients treated with a combination of ampicillin and bismuth (27).

Infection of the gastric mucus and mucosal surface by *H. pylori* may be similar to chronic infections such as endocarditis because of the poor response of leukocytes in these tissues (37) and because *H. pylori* is a fastidious, slowly growing microorganism. Accordingly, it might be predicted that a long course of therapy with bactericidal agents would be the most effective therapy. Both ampicillin and bismuth have been shown to be bactericidal against slowly growing *H. pylori* in broth (28) as well as against slowly growing *H. pylori* attached to cells (27). However, the bactericidal nature of these agents has not been well studied in logarithmic growth phases.

We studied ampicillin and bismuth alone and in combination in both logarithmic and stationary growth phases, using concentrations that would likely be achieved in infected mucosal surfaces in order to evaluate the potential role of bactericidal activities of these agents against actively replicating and nonreplicating strains of *H. pylori*. We found that ampicillin alone is more active against logarithmically growing strains of *H. pylori* than against stationary-phase organisms. In contrast, we found that bismuth alone was less active against some strains in logarithmic phase than against the same strains in stationary phase. It should be noted that in contrast to logarithmic growth, stationary growth was achieved by growing cells at a lower temperature and under ambient air conditions. Therefore, our findings may not be due solely to the effects of lower temperature but may also be due to an increased concentration of oxygen. Which of these factors (perhaps both play a role) is responsible for stationary growth is not of particular importance in this study, so long as stationary growth was maintained for 24 h.

On the basis of the results, we conclude that the activities of antimicrobial agents used singly or in combination against *H. pylori* in both logarithmic and stationary growth phases may be important. The efficacy of double or triple therapy may, in part, be due to the provision by these combinations of antimicrobial activities against both growth phases of *H. pylori*. Other potential advantages of these drug combinations may be related to the prevention of resistance against certain agents. However, development of resistance has not been reported as a problem

for either ampicillin or bismuth. The results of our study would suggest that the increased efficacy of this combination may be due to the targeting of replicating cells by ampicillin while bismuth targets nonreplicating cells. The relatively high rate of recurrence of *H. pylori* infection seen with this combination may be related to our observation that bismuth was minimally inhibitory for one of three strains tested in the stationary growth phase.

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