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Bactericidal Effect of Amoxicillin on *Helicobacter pylori* in an In Vitro Model Using Epithelial Cells

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The treatment of *Helicobacter pylori* with antimicrobial agents has largely been ineffective, and susceptibility results are in disagreement with those obtained by standard in vitro testing. The bactericidal effect of amoxicillin was tested in an in vitro model by using sessile bacteria attached to HEp-2 cells; this bactericidal effect was compared with that against planktonic bacteria. Viable cell counts were performed by standard procedures after 1, 6, and 24 h of contact with the antibiotic at different concentrations. A bactericidal effect (99.9% killing) was observed against sessile bacteria after 24 h with concentrations of only 10, 1, and 0.1 mg/liter, while against planktonic bacteria it was also noted at concentrations of 0.01 and 0.001 mg/liter, and the effect was observed after 6 h with the three highest concentrations. When the results for five strains were studied by analysis of variance at 6 and 24 h, the main variable was the antibiotic concentration, followed by the culture conditions, e.g., planktonic or sessile bacteria, the strain tested, and the time of contact. A decreased pH of 5.4 did not affect the action of amoxicillin. The bactericidal effect of the combination of amoxicillin and metronidazole was additive against sessile *H. pylori*.

Helicobacter pylori is recognized as an important factor in the development of peptic ulcers (11). While peptic ulcer relapse occurs in up to 80% of cases during the year following the first diagnosis, eradication of *H. pylori* has been shown to improve greatly the natural history of the disease. In some studies in which eradication is obtained, the relapse rate is almost nil (25). However, eradication is difficult to obtain. There is no single agent which can achieve eradication in a significant percentage of cases (16). Amoxicillin treatment has given the best results, with approximately 10 to 20% eradication (3). These results contrast with the apparently high susceptibility of *H. pylori* in vitro to most antimicrobial agents, including amoxicillin (8, 10, 13, 15, 20), which indicate that the traditional MIC determination is not adequate for testing this mucosa-associated bacterium.

The concept of biofilm has been proposed by Costerton (6). By studying industrial ecosystems, Costerton (6) showed that attached organisms (sessile bacteria) differ markedly from floating organisms (planktonic bacteria) that are present in the same ecosystem and differ even more markedly from cells that are grown in pure culture in laboratory media. Such differences include growth rate as well as susceptibility to a range of antimicrobial agents.

Taking into account this information, we developed a model to test the bactericidal effect of antibiotics in vitro. This model uses bacteria that are adherent to epithelial cells, in an attempt to mimic the in vivo situation.

MATERIALS AND METHODS

Bacterial strains. The following four reference strains of *H. pylori* were used: CIP 101260 (19), CCUG 19104, CCUG 19107, and CCUG 19110. A wild-type strain (Pylo 112) was also used. These strains were isolated from patients in our laboratory and were maintained frozen at −70°C. The MIC for these five strains was 0.007 mg of amoxicillin per liter and 1 mg of metronidazole per liter. They were grown on Wilkins-Chalgren agar (Oxoid) with 10% human blood under microaerobic conditions (H_2 + CO_2 without a catalyst in a jar; GasPak; BBL).

Cell line. An HEp-2 epithelial cell line was used. The growth medium was Eagle minimum essential medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids.

General conditions of the test. Cells were seeded in 25-cm^2 flasks (Nunc) to obtain a subconfluent monolayer after 24 h of growth (10^5 cells).

A suspension of 24-h-old *H. pylori* was prepared and inoculated onto the HEp-2 cells to obtain a concentration of approximately 5×10^6 CFU per flask (2×10^5 CFU/cm^2).

The cells and the bacteria were incubated for 2 h in a CO_2-enriched atmosphere to allow attachment. The medium and planktonic bacteria were then discarded, and the monolayer was washed three times with phosphate-buffered saline.

Fresh medium containing different concentrations of the antibiotic to be tested was added, and the flasks were incubated in a CO_2-enriched atmosphere at 37°C.

At a predetermined time, the flasks were taken, the medium was discarded, and the cells were washed. The cells were harvested with a rubber policeman in 1 ml of phosphate-buffered saline and disrupted with an Ultraturax homogenizer.

Bacterial counts were performed after geometric dilutions of the suspension were obtained; 0.1 ml of each dilution was inoculated onto each plate in triplicate. The plates were incubated for 7 days in a microaerobic atmosphere, and the plates with 30 to 200 CFU were counted.

The same medium without antibiotics was used as a control in each series. Medium was incubated in the flasks under conditions identical to those used for the samples containing antibiotics.

Antibiotic tested. Amoxicillin (Beecham Laboratories) was tested on sessile bacteria at the following concentrations:

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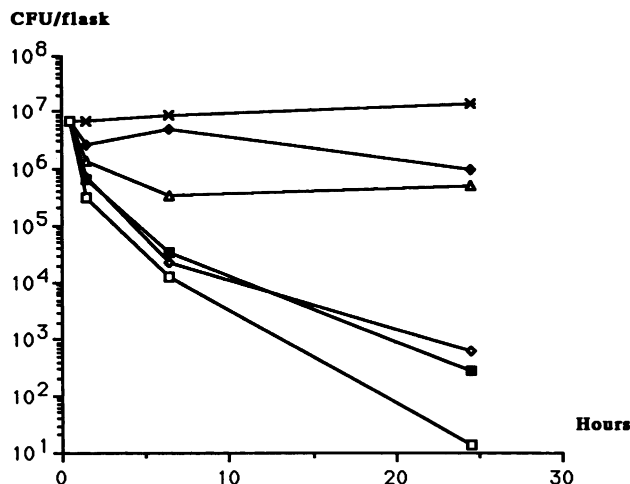

FIG. 1. Bacterial killing of amoxicillin on HEp-2 cells adhering to *H. pylori* CIP 101260 (pH 7.2). Amoxicillin was tested at 0.001 (◆), 0.01 (△), 0.1 (◇), 1 (■), and 10 (□) mg/liter. A control (×) was also tested.

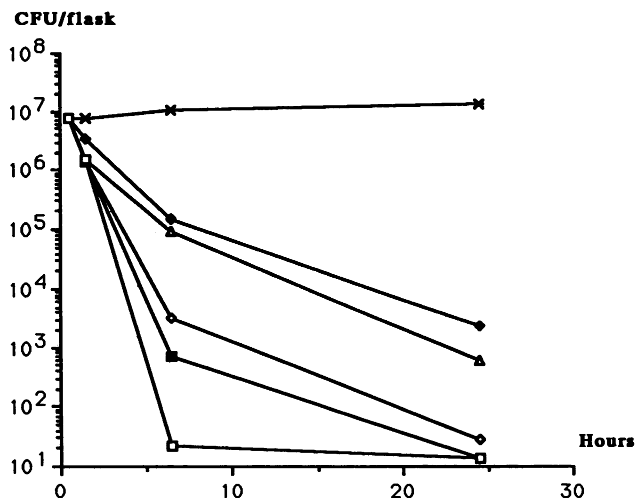

FIG. 2. Bacterial killing of amoxicillin on *H. pylori* CIP 101260 grown in suspension (pH 7.2). See Fig. 1 legend for definitions of symbols.

0.001, 0.01, 0.1, 1, and 10 mg/liter. The bactericidal effect was measured after 1, 6, and 24 h.

The same experiment was performed at pH 5.4 instead of pH 7.2.

In order to determine the role of the association of cell and bacteria, a similar experiment was performed without cells (planktonic bacteria). In this case, 5×10^6 CFU was inoculated directly into the cell culture medium with the antibiotic. After 1, 6, and 24 h, the bacteria were harvested by centrifugation (3,000 × *g*, 10 min) and washed to eliminate the antibiotic and were then suspended in 1 ml of phosphate-buffered saline before being inoculated onto the plates.

A test with gentamicin, a strictly extracellular antibiotic, at a high concentration (20 mg/liter) was performed to define the location of *H. pylori*.

Metronidazole was also tested on sessile bacteria at a concentration of 1 mg/liter with and without 0.01 mg of amoxicillin per liter, in order to determine whether the association had an additive or a synergistic effect.

A postantibiotic effect was tested on sessile bacteria by allowing a 2-h contact of 0.1 mg of amoxicillin per liter with the bacteria and then counting the viable bacteria every 2 h for 12 h and then at 24 h.

Statistical analysis. Statistical analysis was performed by using a general mixed model analysis of variance (BHDP statistical software).

RESULTS

The results obtained with one representative strain (CIP 101260) of *H. pylori* are presented in Fig. 1 and 2.

The action of five concentrations of amoxicillin was tested at pH 7.2 on *H. pylori* adherent to HEp-2 cells under standard conditions. A bacteriostatic effect was found at all concentrations, whereas the control showed an increase in bacteria from 5×10^6 to 1×10^7 CFU per flask. A bactericidal effect (99.9% killing) was observed only with the three highest concentrations after 24 h of incubation (Fig. 1).

In comparison, the bactericidal effect of amoxicillin was present at all concentrations if the bacteria were planktonic rather than sessile (Fig. 2). This effect was observed after 6 h with the three highest concentrations, in contrast to the longer time delay in the former experiment.

The analysis of variance was performed for the five strains together at times of 6 and 24 h. After adjustment for the inoculum, strain differences were present ($P = 0.0022$), while the antibiotic concentration ($P < 0.0001$), the time of contact ($P = 0.019$), and the culture conditions, i.e., sessile or planktonic bacteria ($P = 0.0002$), were significant. The study of the interactions between factors showed that the antibiotic concentration, the time of contact, and culture conditions were independent of the strain used and that the protective effect of the culture conditions, i.e., the sessile form of bacteria, was highest when the antibiotic concentration was the lowest.

When the pH was decreased to 5.4 and monitored, the bactericidal effect was not affected.

Because of the slow growth of *H. pylori* under the conditions used in this study, it was not possible to detect a postantibiotic effect.

The association of amoxicillin and metronidazole showed only an additive effect and not a synergistic effect at both concentrations tested on sessile bacteria (Fig. 3).

DISCUSSION

An important discrepancy has always been noted between the very potent activity of amoxicillin against *H. pylori* when tested in vitro by conventional methods, such as the MIC method, and the results of *H. pylori* eradication in vivo. Eradication is achieved only in approximately 10 to 20% of cases.

One explanation for this discrepancy could be that the MIC method used to test *H. pylori* antimicrobial susceptibility is not relevant. *H. pylori* infection is a mucosal infection, with bacteria lying in the mucous layer and being strongly attached to the cells. This attachment could modify the susceptibility of bacteria to antibiotics. Moreover, *H. pylori* lives in an environment which does not seem to be favorable to phagocytic cells, and therefore, a bactericidal instead of a bacteriostatic effect must be considered.

For these reasons, we extended our in vitro model for the study of adherence (21) to amoxicillin, one of the best

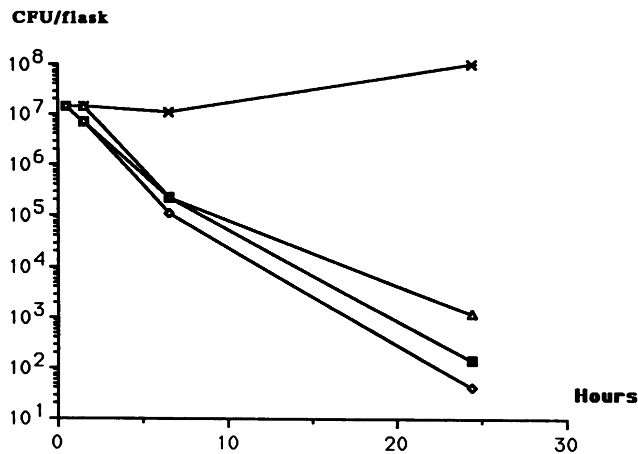

FIG. 3. Bacterial killing of amoxicillin, metronidazole, and their association on *H. pylori* CIP 101260 adherent to HEp-2 cells. ×, control; △, amoxicillin, 0.1 mg/liter; ■, metronidazole, 1 mg/liter; ◇, amoxicillin and metronidazole.

candidates for *H. pylori* eradication. We used HEp-2 cells, an epithelial cell line which harbors the same membrane glycolipid receptor for *H. pylori* as those harbored by the mucous cells of the antrum (17).

Our results indicate that when the bacteria adhere to the cells, the susceptibility of *H. pylori* to amoxicillin is altered. These results were obtained for all the strains tested, and an analysis of variance showed that the culture conditions were an important determinant in the differences in susceptibilities that were observed. The possibility of internalization of the bacteria by the cell was explored. Under the same conditions, gentamicin, a strictly extracellular agent which was used at a high concentration that was only achievable in vitro to test this hypothesis, always killed all the bacteria present.

The mechanism of this resistance to bacterial killing is not known. In a model of *Staphylococcus epidermidis* isolates adherent to stainless steel substrata, Gristina et al. (12) found that strains for which the MBC was less than 0.5 mg of tobramycin per liter showed only a 2-log-unit reduction in bacterial population when the bacterial biofilm was exposed to 10 mg of this antibiotic per liter during 8 h. The same antibiotic was also used in a model of *Pseudomonas aeruginosa* adherent to a urinary catheter. While the MIC for the strain was 0.4 mg/liter, a 12-h exposure to the flow of tobramycin in sterile artificial urine achieved a drop of from only 10^8 to 10^6 viable organisms per cm^2 (22). This was not due to selection of a resistant mutant; the explanation was the production of slime. Costerton and Marrie (7) proposed that the bacterial glycocalyx has a role in resistance to antimicrobial agents. An alternative to this explanation would be a change in the structure of sessile bacteria in comparison with the structure of its planktonic counterparts. It is known that the compositions of microorganisms, and especially components of the outer membrane, are regulated by the nature of the growth environment (2). *H. pylori* has been shown to produce a glycoprotein coat (9). Adherence could enhance the production of this substance, which would act as a penetration barrier for amoxicillin, but in no way is it a true biofilm. By this last hypothesis, the resistance of bacteria would be a growth-related phenomenon (4). The cells within biofilms grow very slowly and resemble stationary-phase cultures. The growth rate is known to be a primary modulation of antibiotic action. This is especially true for β-lactam antibiotics (27). In our experiment, the number of viable cells increased by only 50% during 24 h, but they increased in the same proportion for the sessile and planktonic populations. This result is quite different from the observation made by Prosser et al. (24) with *E. coli* adherent to silicon latex catheters which were in the stationary phase of growth, because the planktonic population increased by 300%, and therefore, this explanation may not be true for *H. pylori*.

In the model described here, we tried to stay close to generally accepted standards of bacterial killing experiments (14, 26). An important parameter, the inoculum size (10^6 to 10^7 CFU), was within the normal range. Bacteria were tested during their growth phase, since it is particularly difficult to obtain *H. pylori* in a stationary but still viable phase and the transformation into nonviable coccoidal forms is very rapid. This large inoculum permitted the testing of the heterogeneity of the population. Because of the design of the study, the eventual adherence of *H. pylori* to plastic, which is a potential problem in the classical bactericidal test, did not have to be taken into account, and so agitation was not performed. The incubation atmosphere was adequate for *H. pylori* growth and is not known to impair the activity of amoxicillin. We used a cell culture medium with 10% fetal bovine serum which allowed the growth of *H. pylori*. The concentrations of amoxicillin were multiples of the MIC, and subculture at different time intervals allowed the determination of either a rapid bactericidal effect (1 and 6 h) or a slow bactericidal effect or eventual regrowth (24 h). Bacterial counts were performed by a standard reference method. The plates were incubated for 7 days to ensure the growth of a maximum number of bacteria. The threshold of detection was less than 10 CFU per flask, since 0.3 ml of a total volume of 1 ml was inoculated.

Few studies have determined the concentration of amoxicillin present in the gastric mucosa. In the study of Cooreman et al. (5), following oral intake of 1 g of amoxicillin, a concentration greater than 0.1 mg/g was present in the mucosa after only 30 min but not at 60 and 90 min. In a similar study, McNulty et al. (18) found concentrations ranging from 0.01 to 0.32 mg/g 90 min after administration of a 500-mg oral dose. These results can explain why disappointing results have been observed in vivo when amoxicillin is used as a single agent; 0.1 mg/liter is bactericidal only after 24 h of contact with the bacteria, and this goal was not achieved in vivo. For this reason, the action of an antimicrobial agent with an additive or synergistic effect against *H. pylori* when combined with amoxicillin is needed.

We tested metronidazole, a compound known to improve the eradication rate when it is combined with amoxicillin (23). An additive effect was noted at both concentrations tested. Research must continue to find an agent with a synergistic effect and, moreover, to which resistance is not easily acquired.

This model is different from the in vivo situation because of the lack of a mucous layer which can modify the antimicrobial activity and also because it is not a dynamic model which reproduces the flow of substances going through the mucosa. However, although it is time-consuming, this model will be useful for studying the action of antibiotics against *H. pylori* in vitro and will serve in avoiding expensive in vivo trials in the same way as biofilms grown on inert surfaces are used to test the susceptibility of bacteria isolated from devices (1, 24). Moreover, it could be used to study the

antimicrobial susceptibilities of other cell-adherent bacteria, such as endocarditis streptococci, by using endothelial cells as support.

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