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New Pharmacokinetic In Vitro Model for Studies of Antibiotic Activity against Intracellular Microorganisms

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The capacity for intracellular growth is an important survival strategy for a large group of common pathogens. *Helicobacter pylori*, the etiological agent for gastritis and duodenal ulcer, has been shown by both in vivo and in vitro studies to have the capacity to invade epithelial cells. In vitro models are used to study the effect of antibiotics on microorganisms. Most investigations are performed in broth culture or on agar plates, but kinetic models for bacteria in broth have been described. We present a new, kinetic model adapted for intracellular pathogens. A glass chamber, with a metal rack fitting Falcon cell culture inserts, was connected to a pump by rubber tubes. Different tube diameters and pump speeds were evaluated, and the assay was designed to mimic the half-lives of the antibiotics in vivo, i.e., 11.5 h for azithromycin, 5 h for clarithromycin, and 1 h for amoxicillin. Monolayers of HEp-2 cells were grown in the inserts for 2 days, after which *H. pylori* (clinical strain 88-23), was added to the system. Internalization was allowed for 12 h, and extracellular *H. pylori* cells were eradicated with gentamicin. The inserts were moved to the glass chamber, containing medium with 12.5 mg of either amoxicillin or azithromycin per liter or 2.4 mg of clarithromycin per liter. This represents 12.5, 50, and 80 times the extracellular minimum bactericidal concentration value, respectively. Samples were taken at 0, 2, 4, 6, 8, and 24 h. The HEp-2 cells were lysed, and intracellular bacteria were counted by plating. Inserts with infected cells grown in drug-free medium were included as controls for each time interval. A 3-$\log_{10}$ reduction of *H. pylori* was achieved in the experiments with azithromycin, and a 4-$\log_{10}$ reduction was achieved in the clarithromycin experiments, while no intracellular effect was seen when amoxicillin was used. The antibiotic concentrations at the sampling intervals were 12.5, 3.1, 0.8, 0.2, 0.05, and 0 mg/liter for amoxicillin; 12.5, 11.5, 10, 9, 8, and 3 mg/liter for azithromycin; and 2.4, 1.8, 1.4, 1, 0.8, and 0 mg/liter for clarithromycin. This new model for pharmacokinetic studies provides a useful tool, with applications for a broad range of microorganisms.

In vitro models are important tools when studying the effects of antibiotics on bacteria. Existing models have the obvious disadvantage of measuring the static antibacterial activity in broth culture or on agar plates. Attempts have been made to construct in vitro models that mimic the serum concentration time curve in humans, and in an attempt to simulate the in vivo situation, pharmacokinetic models have been designed for bacteria in broth culture (4, 22, 25). The available models have so far been designed for studies of antibiotic activity on extracellular bacteria and exclude bacteria with an intracellular growth pattern from study.

Facultative, intracellular growth is now accepted as a strategy for survival, as is the importance of facultative, intracellular parasitism in the pathogenesis of a disease (24). As early as 1916, Rous and Jones suggested that intracellular organisms may escape the host's immune response (30). This evasive action has been described for enteric bacteria, such as *Salmonella* spp. (10), *Campylobacter* spp., *Shigella* spp. (18), *Yersinia* spp., and *Escherichia coli* (33). Various other bacteria, i.e., *Proteus mirabilis* (5), *Haemophilus influenzae* (11), group B streptococci (31), and *Listeria monocytogenes* (12), also exhibit an invasive ability. *Helicobacter pylori*, the causative agent of chronic gastritis and duodenal ulcer, has been observed to penetrate epithelial cells when studied both in vivo and in vitro (9, 28). This phenomenon may be a survival strategy for the organisms in order to escape antimicrobial treatment or other hostile elements in the gastric environment (15). It has been shown that treatment strategies including intracellularly active antibiotics are much more effective. Clarithromycin, a macrolide with high concentrations both extracellularly and intracellularly, is now included in many treatment regimens for *H. pylori*, with eradication rates reaching 90 to 95%. This is a great improvement compared with earlier recommended dual treatment regimens with amoxicillin, which cleared only 50 to 60% of the infections (2, 17, 19, 20).

The activity and possible applications of new antimicrobial drugs, or of drugs in new combinations, need to be evaluated in vitro before clinical investigations can take place. There are several in vitro models designed for intracellular pathogens with different cell types. We previously developed an easy and straightforward model for the study of antibiotic activity on intracellular *H. pylori* (16). However, these static models have poor correlation to the situation in vivo. Therefore, in order to reflect the clinical situation, a new pharmacokinetic model for the study of intracellular growth was constructed, in which antibiotic half-lives in vivo could be mimicked.

The aim of this study was to compare the effects of amoxicillin, azithromycin, and clarithromycin on intracellular *H. pylori* in our new model. Amoxicillin and clarithromycin are commonly used for treatment of *H. pylori* infections. Amoxicillin is mainly found in extracellular fluid, and the intracellular effect is minute (1, 32), while clarithromycin is present both intra- and extracellularly. Azithromycin, on the other hand, accumulates in cells and tissues (13, 23) and is found only to a certain degree in extracellular fluids.

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MATERIALS AND METHODS

Equipment and assay development. A glass chamber with two exits and a metal rack fitting Falcon cell culture inserts (Becton-Dickinson, Franklin Lake, N.J.) were constructed (Fig. 1). The inserts were 1.05 cm in diameter and incorporated Cyclopore polyethylene terephthalate track-etched membranes with pores measuring 0.45 μm. The glass chamber was connected to a pump (C6-T SC; Alitea AB, Stockholm, Sweden) by santoprene tubes (Aliprene; Alitea AB). A magnet was placed in the bottom of the glass chamber, which was placed on a magnetic stirrer to achieve an even concentration throughout the medium.

The pump was adjustable from 10 to 100 rpm. To achieve the desired antibiotic half-lives inside the glass chamber, tubes with different diameters were tested at different pump speeds. Spectrophotometric A_{430} values were measured at different intervals as a bromthymol blue (BTB) solution (0.04%) was diluted with water. Graphs were plotted to visualize the dilution of BTB with time, and the half-lives with different tubes and pump speeds could be estimated. Three different setups were chosen for our experiments.

In the azithromycin and clarithromycin experiments, the experimental half-lives were verified; samples were taken from the medium inside the inserts at 0, 2, 4, 6, 8, and 24 h. These were stored at −20°C and analyzed at the end of the experiment. A bacterial suspension of *E. coli* MB 3804, which was sensitive to azithromycin and clarithromycin, was poured onto tryptone-glucose agar plates (5 g of Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 1 g of glucose, 2.5 g of Bacto yeast extract [Difco], 2 g of Na_2HPO_4, 15 g of agar [BBL; Beckton-Dickinson and Co., Cockeysville, Md.] per liter), and wells were made in the agar plates. Three agar plates were prepared in each experiment. Forty microliters of known antibiotic concentrations (8, 4, 2, and 1 mg/ml) was added to four of the wells on each agar plate, and to the remaining wells, 40 μl of sample medium from the different time intervals was added. The agar plates were incubated overnight at 37°C, and the clear zones around the wells were measured (limit of detection, 0.1 mg/liter). The coefficients of variation for samples assayed the same day was 0.1 to 1.7% for azithromycin and 0.4 to 1.7% for clarithromycin. Between days, values were approximately the same. A mean value was calculated for each sample, and a standard curve was plotted from which the sample concentrations could be estimated (correlation coefficient, >0.98). The experimental half-lives were deduced from a graph with the achieved azithromycin and clarithromycin concentrations plotted against time.

Cell culture. A human epithelial cell line, HEp-2 (ATCC CCL 23), was cultured in a medium consisting of RPMI 1640 (Gibco BRL, Life Technologies, Paisley, Scotland), 10% fetal calf serum, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 2 mM glutamine, and 0.05% $NaHCO_3$ at 35°C and 5% CO_2. The cells were washed, treated with trypsin, and seeded into Falcon cell culture inserts at a density of 1×10^5 to 2×10^5 cells per ml.

Preparation of bacteria. *H. pylori* clinical strain 88-23 (kindly provided by M. Blaser, Nashville, Tenn.) was grown on chocolate agar plates (Columbia II agar Base BBL; Becton-Dickinson and Co.) with 10% horse serum and 8.5% horse blood). Growth was performed at 37°C in a humid atmosphere under microaerophilic conditions (7% CO_2 and 87% N_2). Seven different strains of *H. pylori* were compared for invasive abilities, and the clinical strain 88-23 was chosen because of its greater invasiveness (Table 1). The bacteria were grown in brucella broth with 10% fetal calf serum for 3 days before they were introduced to HEp-2 cells. The purity of *H. pylori* cultures was verified in each experiment by Gram staining and by biochemical characteristics, i.e., positivity in urease, catalase, and oxidase tests. Bacteria were harvested by centrifugation and were resuspended in the RPMI medium mentioned above to an optical density at 530 nm of 0.5, which corresponds to approximately 10^8 CFU per ml, as determined in each experiment by viable counting.

Preparation of antibiotics. Amoxicillin (kindly donated by Astra, Södertälje, Sweden) was dissolved in 0.1 M phosphate buffer, pH 8.0, to a concentration of 10 mg/ml and was subsequently diluted to 12.5 mg/liter in the RPMI medium, adjusted to pH 7.4. Amoxicillin has a half-life in vivo of approximately 1 h (21). Consequently, the model was sent to mimic this characteristic.

A stock solution of azithromycin (kindly provided by Pfizer, Inc., New York) was prepared by dissolving 10 mg of the drug in 1 ml of methanol. It was diluted to an initial concentration of 12.5 mg/liter in the same manner as that described for amoxicillin. Azithromycin has a mean terminal elimination half-life of 11 to 57 h in human serum, depending on how the drug is administered and on how long the period of time is during which the half-life is determined (29). For convenience, we chose a half-life of 11.5 h in this study.

Clarithromycin (kindly provided by Abbott Laboratories, North Chicago, Ill.) was prepared by dissolving 10.2 mg (potency 0.976) in 4 ml of methanol and was diluted with 6 ml of 0.1 M phosphate buffer (pH 6.5). A half-life of 5 h was used, and the initial concentration of drug was 2.4 mg/liter (35). All drugs were prepared immediately before each experiment.

Gentamicin (Gensumicin; 10 mg/ml; Roussel, Denham Uxbridge, United Kingdom) was added to the RPMI medium to a final concentration of 50 mg/liter.

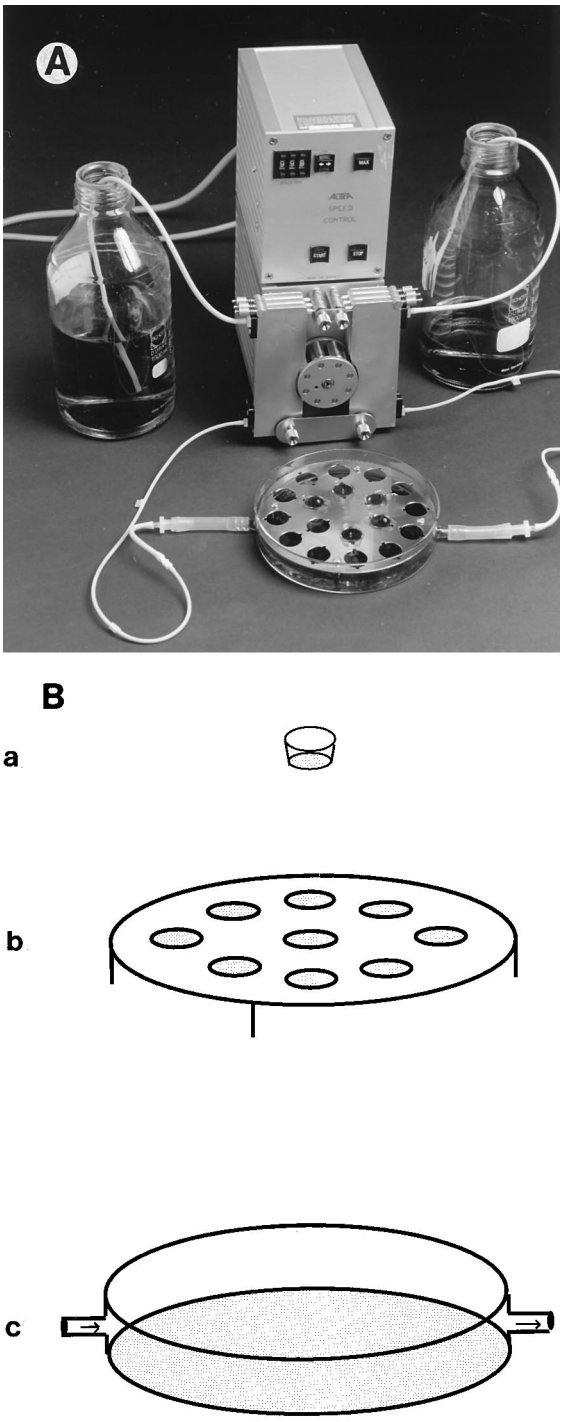

FIG. 1. (A) The Cb-T SC pump, connected to the autoclavable parts of the equipment, i.e., the glass chamber with metal rack and santoprene tubes. (B) The different parts of the construction, including cell culture insert (a), rack (b), and glass chamber (c).

Sensitivity of *H. pylori* to amoxicillin, azithromycin, and clarithromycin. The susceptibility of strain 88-23 to the drugs used was determined by adding 20 μl of bacterial suspension at an optical density at 530 nm of 0.5 to twofold serial dilutions of each antibiotic in 2 ml of brucella broth (pH 7.4) and incubating for 3 days. MIC was defined as the lowest concentration inhibiting visible growth. Minimal bactericidal concentrations (MBC) were determined by plating 100 μl of 10^1 and 10^2 dilutions from each antibiotic concentration onto chocolate agar

TABLE 1. Internalization capacity of *H. pylori* strain[a]

Strain	Intracellular concn (CFU/ml)	Intracellular/extracellular bacteria (%)
88-23	8.7×10^3	2.7×10^{-3}
H:72	5.2×10^2	5.8×10^{-4}
NCTC 11637	3.5×10^3	1.9×10^{-3}
K444-2010	5.7×10^2	2.5×10^{-3}
K418-5023	6.5×10^3	6.5×10^{-4}
SVA-40	1.1×10^3	7.3×10^{-4}

[a] Strain 88-23 penetrated the HEp-2 cells to the highest extent, and this strain was used in all experiments. Approximately 10^5 cells were exposed to the bacterial inoculum, which varied at approximately 10^8 CFU/ml, and all cells seemed to ingest at least one bacterium ($10^6/10^5$ cells).

plates. MBC was the lowest concentration to result in killing of 99.9% of the inoculum.

The bacteria proved sensitive to the antibiotics used before and after each experiment (tested by the E test).

Exposure of HEp-2 cells to *H. pylori*. Inserts with HEp-2 cells grown overnight were exposed to *H. pylori* suspended in RPMI medium to a concentration of 10^8 CFU per ml, and internalization was allowed for 12 h at microaerophilic conditions.

Extracellular *H. pylori* were eradicated by washing the wells four times with RPMI medium containing 50 mg of gentamicin per liter and subsequently incubating the exposed cells for 2 h in the same medium. One hundred microliters from the cell growth medium was plated on to chocolate agar plates to verify that no extracellular surviving *H. pylori* cells were present, and the cell culture inserts were washed five times with phosphate-buffered saline (PBS) before the experiments were continued.

Study of the effects of amoxicillin, azithromycin, and clarithromycin on intracellular *H. pylori*. The cell culture inserts were installed in the glass chamber, and RPMI medium with 12.5 mg of either amoxicillin or azithromycin per liter or with 2.4 mg of clarithromycin per liter was added. Controls, i.e., inserts with *H. pylori*-exposed HEp-2 cells, were grown separately in RPMI medium without antibiotic supplement. After 0, 2, 4, 6, 8, and 24 h, two samples were removed from the glass chamber and from the controls. The cells were washed three times with PBS and incubated with trypsin for 5 min. The cells were removed from the membranes with a cell scraper, and the solutions were transferred to Eppendorf tubes. A five-min centrifugation at 15,800 × *g* and a washing step with PBS to remove residual trypsin were followed by 10 min of lysis with distilled water. After centrifugation at 15,800 × *g* for 5 min, the pellets, consisting of bacteria and cell debris, were dissolved in 1 ml of brucella broth. One hundred microliters from each sample was plated onto chocolate agar plates, which were incubated for 3 days before the colonies, i.e., the viable intracellular bacteria, were counted (limit of detection, 10 CFU per ml).

RESULTS

Evaluation of equipment. With a tube diameter of 0.508 mm and at 15 rpm, a half-life of 11.5 h was achieved inside the cell culture inserts, and by using tubes measuring 1.65 mm at 25 rpm, a half-life of 5 h could be accomplished. In order to achieve half-lives of less than 3 h, the inserts had to be punctuated on opposite sides. This was necessary for the amoxicillin experiments, and a half-life of 1 h was achieved with a tube diameter of 1.65 mm at 16.5 rpm.

MIC and MBC in broth. The MIC and MBC for amoxicillin were 0.5 and 1.0 mg/liter, respectively, the values for azithromycin were 0.125 and 0.25 mg/liter, respectively, and those for clarithromycin were <0.0035 and 0.03 mg/liter, respectively.

Intracellular activities of antibiotics. A 3-$\log_{10}$ reduction of *H. pylori* was achieved after 24 h of incubation with azithromycin. The antibiotic concentrations at the different time intervals, as evaluated by the agar test, were 12.5, 11.5, 10, 9, 8, and 3 mg/liter, respectively. Clarithromycin showed an even better killing with a 4-$\log_{10}$ reduction of intracellular bacteria. The antibiotic concentrations were the same when deduced from the graphs in the BTB dilution experiments, and this good correlation made us rely on the data from the BTB experiments in the amoxicillin experiments.

No intracellular effect was seen in the experiments with amoxicillin, and the graph is almost identical to the graph of the controls. The antibiotic concentrations correspond approximately to 12.5, 3.1, 0.8, 0.2, 0.05, and 0 mg/liter for the different time intervals (Fig. 2 and 3).

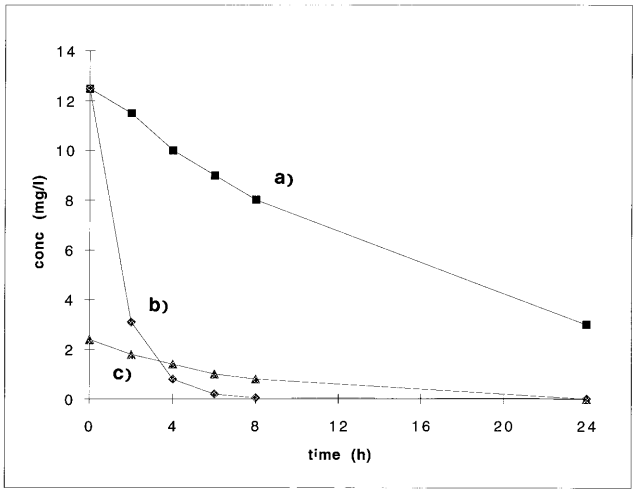

FIG. 2. Graphs for determination of half-lives inside the cell culture inserts for azithromycin (a), amoxicillin (b), and clarithromycin (c). The results are based on BTB dilution experiments (a) or measurements by the agar method (b and c).

DISCUSSION

Although our knowledge of pharmacokinetics and distribution of drugs in tissue has increased in the past 2 decades, many factors involved remain to be investigated. A better understanding of the complex interaction between antibiotics, bacteria, and the host is obtained in animal models and by clinical investigations. Even if pathogen-cell interactions are not identical in the laboratory milieu and in the in vivo situation, an in vitro screening model reflecting the clinical pharmacokinetic distribution could be useful at an initial screening phase of drug evaluation. The use of such assays should hopefully limit the number of animal experiments for this purpose.

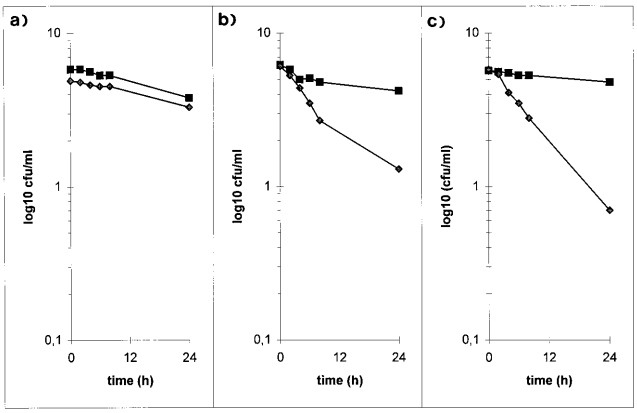

FIG. 3. Intracellular activities on intracellular *H. pylori* cells of amoxicillin (a) (■, control; ◆, amoxicillin [12.5 mg/liter at time 0]), azithromycin (b) (■, control; ◆, azithromycin [12.5 mg/liter at time 0]), and clarithromycin (c) (■, control; ◆, clarithromycin [2.4 mg/liter at time 0]). The results for amoxicillin are based on mean values from two experiments, and the azithromycin and clarithromycin results are based on three experiments each.

The cell culture inserts used in our model have pores of 0.45 μm in diameter which prevent bacteria or cells from spreading between samples. This barrier between samples makes it feasible to use a variety of cells, including nonadherent cell types (i.e., macrophages and polymorphonuclear leukocytes), polarized cells, and human-derived tissue cells in the assay and extends the field of applications. Treatments for parasites such as *Plasmodium falciparum* and *Toxoplasma gondii* that need to be grown in erythrocytes and macrophages, respectively, could be evaluated. Results achieved with laboratory cell lines could be verified by repetition of experiments with relevant, human-derived cells. A suitable application regarding facultative intracellular pathogens would be to include extracellular and intracellular bacteria in the same assay. Compared with our study of exclusively intracellular *H. pylori*, such an approach would better correspond to the in vivo situation.

Different applications for use of this model are currently being investigated. This model has also been used for culture of biopsies. We hope to be able to improve the viability of the biopsies with a constant flow of fresh media.

In the present study, the extracellular concentrations of azithromycin were 50× the MBC in the beginning of each experiment and 4× the MBC after 24 h of incubation. Theoretically, the intracellular concentrations would be even higher. Azithromycin was used in the experiments to compare the results with the static model. Since we had achieved a significant reduction of bacteria with 10 mg of azithromycin per liter and merely no effect with 1 and 0.1 mg/liter in our previous model, this concentration was preferred even though the peak concentration in serum was much lower, i.e., 0.45 mg/liter (6). The area under the concentration-time curve value for azithromycin is high compared with what has been measured in vivo, i.e., 207.4 versus 3.39 mg/liter · h 72 h after administration of a 500-mg single dose (6).

Clarithromycin was added to the system with a concentration of 80× the MBC and was diluted during the experiment to nearly 0 mg/liter. We had not used clarithromycin in any model before, and this drug was included since it is commonly used for treatment of *H. pylori*. Whitman et al. reported a peak concentration in serum of 2.4 mg/liter (35), which is why we chose this concentration. The area under the concentration-time curve was 17.2 mg/liter · h, which is similar to in vivo observations (17.4 mg/liter · h after a 400-mg single dose) (29).

During the 24 h of the experiments, the amount of bacteria decreased to $1/10^3$ and $1/10^4$ of the inoculates with azithromycin and clarithromycin, respectively. Longer incubation times might give an effective killing, i.e., 99.9%, of intracellular *H. pylori*, which seems to be necessary in the clinical situation.

We did not measure intracellular concentrations in these experiments, since our intention was to establish an easy and useful model with possible adjustment of antibiotic half-lives, in which the activity of the antibiotic is reflected by the numbers of surviving bacteria. There are many different factors that influence the intracellular efficacy of the drug apart from intracellular concentration. It is important to know which compartment of the cell accumulates the drug and whether the internalized microorganisms reside in the same intracellular space. The effect of the physiochemical milieu on the metabolic state of the drug and on the organism is another determining factor that must not be overlooked (8). Apart from the factors already mentioned, facultative intracellular microorganisms may be in a resting phase or have a less-active metabolism. If so, all drugs targeting the protein synthesis would have a poor effect on these bacteria. Interactions between the bacteria and the cell may also affect the activity. The fairly slow effect of azithromycin further implies a slow metabolism of the bacteria in this situation. Intracellular *H. pylori* cells reside most likely in inclusions in the cytoplasm. A distribution of azithromycin in other compartments could also explain our results. Clarithromycin is more effective than azithromycin in curing *H. pylori* infections in vivo, and our in vitro results are in accordance with the clinical situation.

It has been established that *H. pylori* can convert to a coccoid form in response to stress factors in the environment. We have noted such conversions intracellularly (data not shown). Antimicrobial agents have been shown to evoke this form (3), and although the viability of this round form is doubtful, the possibility remains that it could be a further explanation of poor antibiotic efficacy. The results of the azithromycin experiments were similar to the results previously achieved in the static in vitro model. More bacteria are internalized in the new assay, and the viability of the intracellular bacteria is increased compared with that of the previous model (16).

Amoxicillin enters the cell by diffusion and is found free in the cell cytoplasm. The lack of intracellular accumulation may be explained by an active outward transport (34). The extracellular concentrations were 12.5× the MBC in the beginning of each experiment and close to zero when the experiment was terminated. The peak concentration of amoxicillin in serum is reported by some authors to be 7.4 mg/liter (14, 27) and by others to be 10.8 mg/liter (7). Gordon et al. show an individual variation of 5.17 to 12.20 mg/liter (14). Thus, the concentration used is slightly higher than the values achieved in the clinical situation. Obviously, the intracellular concentrations never reached bactericidal levels during the experiment, and no effect on intracellular *H. pylori* could be seen.

The area under the concentration-time curve was 18.0 mg/liter · h which is less than than the in vivo results, i.e., 49.3 mg/liter · h after a 250-mg single dose (26).

A future development of these experiments will be to screen combinations of antibiotics together with a proton pump inhibitor, such as omeprazole or lansoprazole, which is commonly included in anti-*H. pylori* treatments, and to monitor eventual emergence of antibiotic resistance. Experiments are already in progress in which antibiotic dosing conditions are simulated.

Most antibiotics are screened for their activity on extracellular bacteria, which does not mirror the true clinical situation, since many factors influence the efficacy of a drug in vivo. We have reported a new, kinetic in vitro model in which antibiotic half-lives can be mimicked. We have applied this model to intracellular *H. pylori* and compared the effects of two antibiotics with different mechanisms of action.

This model offers an alternative to animal models, and as a screening model it is a cost-effective alternative. Factors such as dose dependence and intervals between doses can be studied, and we believe it will be a useful tool in basic antibiotic research.

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