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Bactericidal and Morphological Effects of Amoxicillin on *Helicobacter pylori*

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The growth kinetics of *Helicobacter pylori* after it has been exposed to amoxicillin have been investigated in conjunction with studies of cell morphology. A potent bactericidal effect was observed at concentrations 10-fold higher than the MIC, but this was accompanied by an increase in the residual numbers of coccoid forms observed. In the presence of 10 μg of amoxicillin per ml, these forms could be detected as rapidly as 6 h after exposure to the antibiotic. Although the clinical relevance of coccoid forms remains unknown, such forms should be considered when potential anti-*Helicobacter* agents are tested in vitro.

Helicobacter pylori exists in several morphologically distinct forms, including nonculturable coccoid forms (3, 5). Coccoid formation has been attributed to environmental stress, including nutrient deprivation, extended incubation, accumulation of metabolic products, pH alteration, and exposure to antimicrobial agents (1, 3, 5). The last may be of major importance when therapy is considered, but the activity of agents in vitro is often determined only by susceptibility tests, e.g., agar dilution, in which the morphological status of the organism is unknown. Recent studies have demonstrated that amoxicillin at concentrations close to the MIC can result in the development of coccoid forms after 72 h of incubation (2). We describe observations on the time course of the bactericidal and morphological effects of a range of concentrations of amoxicillin on *H. pylori.*

A broth cultivation technique similar to that described previously by Iwahi and coworkers (4) was used. Briefly, stock cultures of *H. pylori* NCTC 11637 were inoculated into 200 ml of Brucella broth (BBL) with 2.5% fetal calf serum (Gibco) in 500-ml sterile tissue culture flasks. After 16 h of microaerobic incubation (Gaspak jars and Campypaks; BBL) with gyration at 100 rpm, the culture (approximately 5 $\log_{10}$ CFU/ml) was divided into 36-ml aliquots in 80-cm^2 tissue culture flasks, and 4-ml volumes of fresh medium or amoxicillin (SmithKline Beecham Pharmaceuticals, Worthing, England) prepared in broth to give concentrations of 0.01, 0.1, 1.0 and 10 μg/ml were added. The MIC of amoxicillin for *H. pylori* NCTC 11637, as determined by agar dilution, was 0.015 μg/ml. The cultures were reincubated as described previously, and at times from 0 to 36 h, 0.5-ml volumes were removed for the enumeration of viable bacteria, measurement of residual antibiotic concentration, and electron microscopy. The viability was determined by a modified Miles-Misra technique of preparing serial 10-fold dilutions in phosphate-buffered saline (PBS) and inoculating 20-μl samples in triplicate onto Mueller-Hinton agar (Oxoid Ltd.) supplemented with 7% defibrinated horse blood. Colonies were counted after 3 days of incubation at 37°C under microaerophilic conditions and were expressed as CFU per milliliter. Samples, taken at random, were also plated onto nonselective media to determine purity, and organisms other than *H. pylori* were not detected. A large-plate agar diffusion technique was used to assay amoxicillin concentrations, with *Micrococcus luteus* NCTC 8340 being used as the assay organism. Samples taken at intervals up to 24 h of incubation were diluted, as necessary, in PBS and assayed in duplicate against standards of amoxicillin prepared in PBS or Brucella broth over a concentration range of 0.008 to 1.0 μg/ml. The correlation coefficients for the regression lines of the standard solutions were not less than 0.997.

Bacterial samples were fixed for microscopy with 2.5% (vol/vol) gluteraldehyde in 100 mM sodium cacodylate buffer. Cells were concentrated by centrifugation, and samples were removed for examination by differential interference contrast light microscopy. Slides were visually assessed for the relative abundances of the cell types present, with an arbitrary scale (−, scarce; +, few cells; and ++, numerous cells) being used to rate the slides. To assist in identification, selected samples were also negatively stained with 1% uranyl acetate for transmission electron microscopy.

The bactericidal studies (Fig. 1) show that at a concentration of 0.01 μg/ml, amoxicillin had very little effect on the growth of *H. pylori* NCTC 11637 and that the bacterial numbers recovered were similar to those for the control (no antibiotic). Amoxicillin at 0.1 μg/ml produced a relatively static response, with counts of approximately 5 $\log_{10}$ CFU/ml throughout the experimental period. At an amoxicillin concentration of 1.0 μg/ml, bacterial numbers were approximately 1 log lower than those for the control at 6 h, but at 12 h, a 5-log reduction was seen. Following exposure of the bacteria to 10 μg of amoxicillin per ml, numbers were below the level of detection (1.69 $\log_{10}$ CFU/ml) by 6 h and remained so for the duration of the

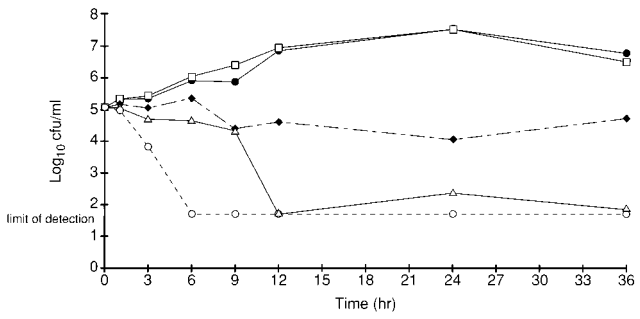

FIG. 1. Bactericidal effect of amoxicillin against *H. pylori* NCTC 11637 (□, control; ●, 0.01 μg/ml; ◆, 0.1 μg/ml; △, 1.0 μg/ml; ○, 10 μg/ml.)

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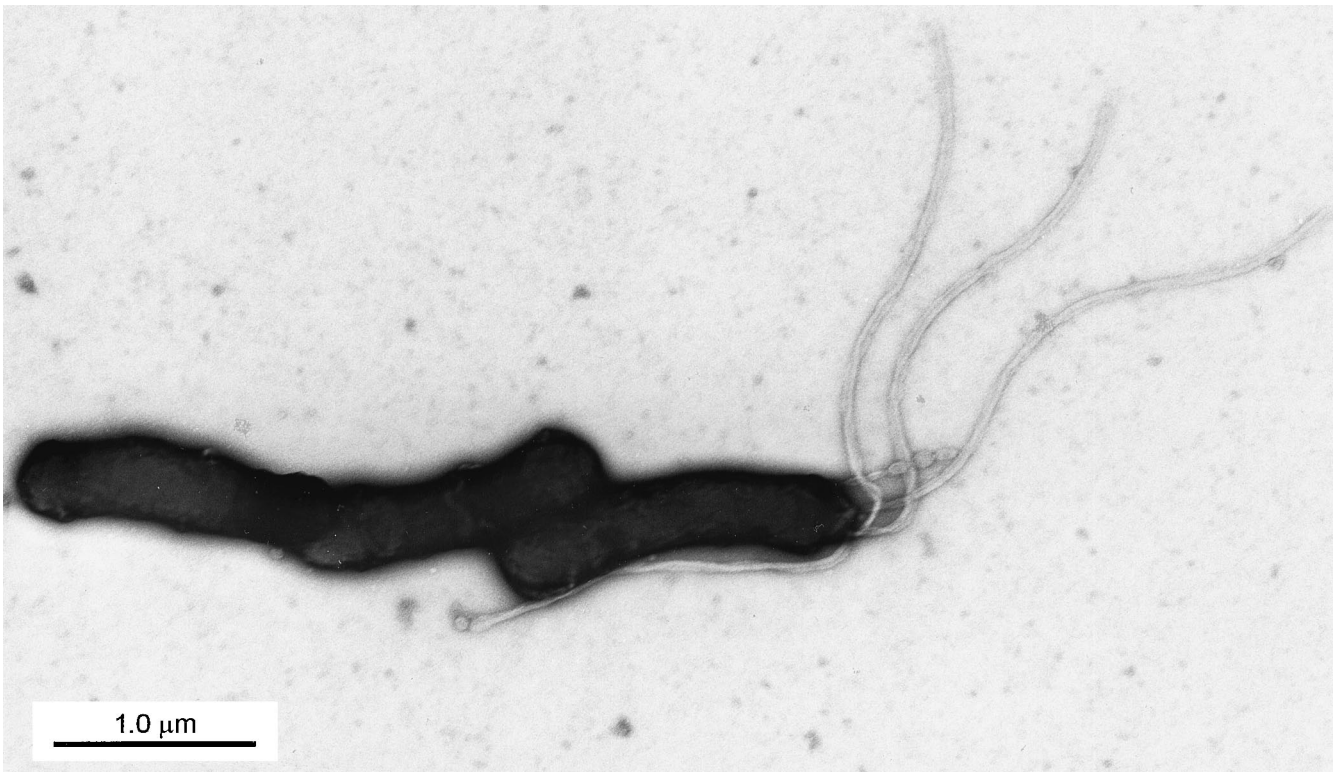

FIG. 2. Electron micrograph of *H. pylori* NCTC 11637 (helical form).

experiment. Samples taken for the measurement of antibiotic concentrations showed amoxicillin to be stable over the period tested (data not shown).

Microscopic analysis of samples showed that, in general, cell numbers appeared low, even following concentration by centrifugation. At 0 h, the culture contained both rod and coccoid forms (Fig. 2 and 3). However, following the addition of fresh broth, rod forms of *H. pylori* with characteristic sheathed fla-

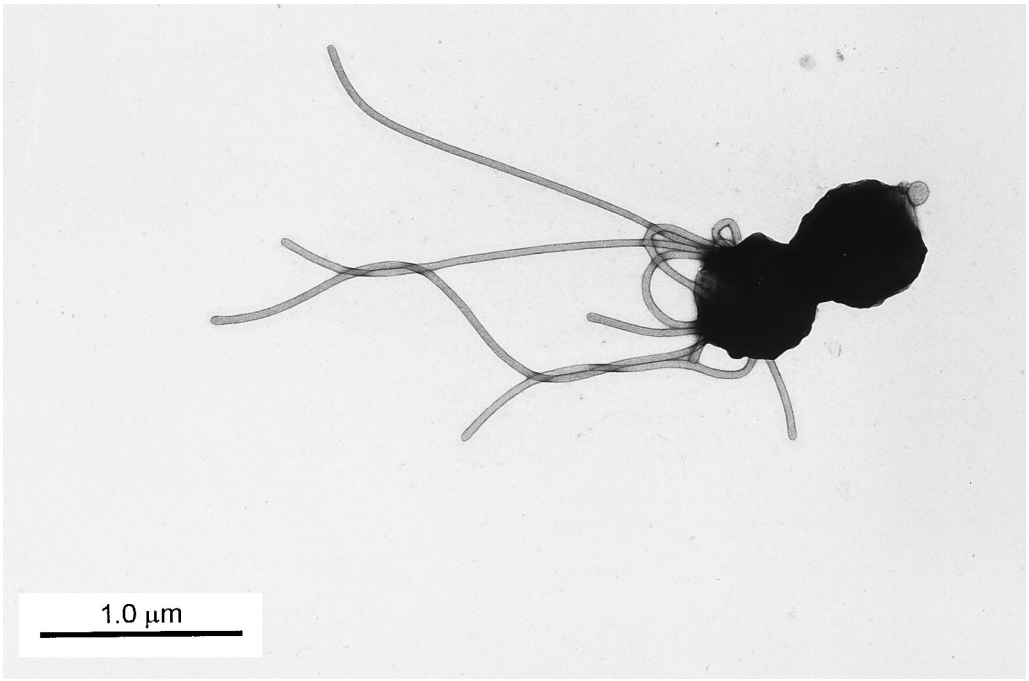

FIG. 3. Electron micrograph of *H. pylori* NCTC 11637 (coccoid form).

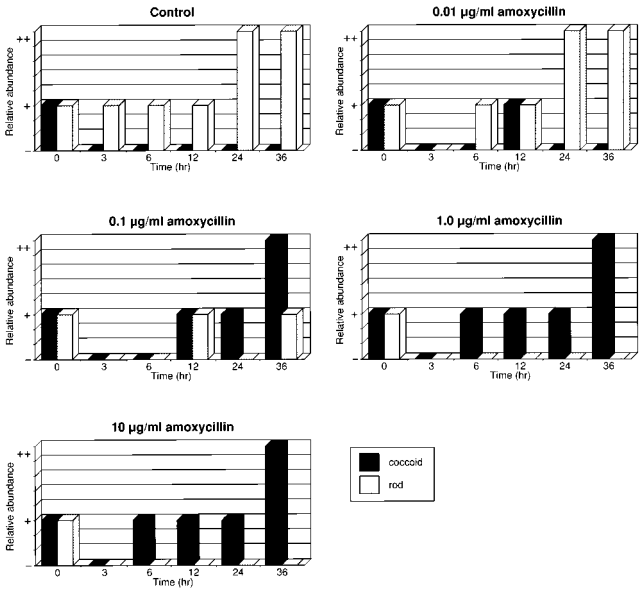

FIG. 4. Relative abundances (−, scarce; +, few cells; ++, numerous cells) of rod and coccoid forms of *H. pylori* NCTC 11637 over a 36-h period in the presence of various concentrations of amoxicillin (0 to 10 μg/ml).

gella were most evident in samples taken from the control broths and from those exposed to 0.01 μg of amoxicillin per ml. The rod forms showed a marked increase in numbers with time (Fig. 4), in accord with an increase in viable bacterial numbers recovered. Coccoid forms were not seen in any great numbers in these samples. This contrasted with the situation for bacteria exposed to concentrations of amoxicillin above 0.01 μg/ml, in which, from 6 h onwards, coccoid forms appeared with greater frequency and rods were less evident. At amoxicillin concentrations of greater than 1.0 μg/ml, coccoid forms dominated the samples.

Results from the bactericidal studies detailed here confirm that amoxicillin has a potent effect against *H. pylori* in vitro. However, when these results are taken in conjunction with the morphological observations, it is apparent that as viable bacterial numbers decreased, the emergence of coccoid forms increased. These data are in agreement with those of Bode et al. (2), who showed a marked increase in the numbers of coccoid forms 72 h after exposure of the organisms to low concentrations (0.05 μg/ml) of amoxicillin. They are particularly important in demonstrating the rapidity with which these forms appear, even at concentrations of amoxicillin that are in excess of 500-fold higher than the measured MIC. Coccoid forms, although metabolically quiescent, have been shown to retain important life functions (1, 6, 8, 9) and have therefore aroused speculation that they may assume a significant role in therapy failure and the recrudescence of infection (2, 3, 7). *H. pylori* is susceptible to most antibiotics in vitro, including amoxicillin, but these agents per se have little effect in the clinic. For successful therapy, it may be essential not only to eliminate the helical and rod form but also to rapidly suppress and/or destroy the coccoid form. In conclusion, our observations suggest that susceptibility testing alone may not be sufficient to provide evidence of the clinical potential of anti-*Helicobacter* agents and indicate the need for further examination of the significance of the morphological status of the organism.

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