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Coccoid and Spiral *Helicobacter pylori* Differ in Their Abilities To Adhere to Gastric Epithelial Cells and Induce Interleukin-8 Secretion

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Helicobacter pylori **exists as an actively dividing spiral form and a nonculturable, but viable, metabolizing coccoid form. Both forms are present in the stomach, but their relative pathophysiologic significances are unknown. Here we show that the coccoid form of *H. pylori*, in contrast to the spiral form, binds poorly to gastric epithelial cells and induces little, if any, interleukin-8 secretion by these cells.**

Helicobacter pylori is associated with gastritis and peptic ulcer disease and may be a risk factor for gastric carcinoma. The organism exists in two forms, an actively dividing spiral form and a coccoid form (reviewed in reference 6). The latter is nonculturable but alive and metabolically active, since it synthesizes DNA (3, 21), accumulates polyphosphates as an energy and phosphorus source (3), and reduces tetrazolium dyes (13). The coccoid form has been shown to result from extended incubation in water (21), and a waterborne route of infection may exist in developing countries (15). Conversion from the spiral to the coccoid form can be induced by increased oxygen tension (4, 6), alkaline pH (4, 6, 17), increased temperature (21), or extended incubation (4, 19), as well as by treatment with omeprazole (6) or antibiotics such as amoxicillin (1, 3). Both forms can be found in the human stomach (7) and duodenum (18). Moreover, coccoid *H. pylori* is found attached to severely damaged gastric epithelial cells (16). Coccoid bacteria are prevalent around margins of gastric tumors and can be identified in 93% of biopsy specimens from patients with *H. pylori*-associated adenocarcinoma (7). However, the role of the coccoid form in pathogenesis remains unclear (5, 11). Therefore, we compared the two forms with respect to the ability of the bacteria to induce interleukin-8 (IL-8) secretion (9, 10, 14, 23) and to adhere to human gastric epithelial cells (20).

Two strains (SD 4 and SD 14) of *H. pylori* were isolated from gastric biopsy specimens of confirmed duodenal ulcer patients. These studies were approved by the Human Subjects Committee at the University of California, San Diego. Bacteria were recovered on Columbia agar supplemented with 7% laked horse blood, 2% IsoVitaleX (BBL, Cockeysville, Md.), and Dent supplement (Oxoid-Unipath, Basingstoke, England). Both strains were *cagA* and *vacA* positive, as indicated by appropriately sized bands on agarose gels following PCR amplification of bacterial DNA (primers were based on sequences described in references 8 and 22). Bacteria were subsequently expanded in liquid cultures with RPMI medium that was supplemented as previously described (19). Liquid and agar cultures were incubated at 37°C under microaerophilic conditions by using Campy Paks (BBL). *H. pylori* grown in liquid cultures under these conditions for 3 to 5 days was >90% spiral. To obtain coccoid *H. pylori*, incubation of liquid cultures was continued for >10 days in a Campy Pouch; after this >90% of the bacteria were coccoid. Total bacteria were counted in a Petroff-Hausser chamber with a dark-field microscope, and the percentage of coccoid bacteria was determined by staining with Wright-Giemsa stain. The human gastric epithelial cell lines AGS and Kato III were obtained from the American Type Culture Collection (Rockville, Md.) and were maintained in RPMI medium supplemented with 10% fetal calf serum. All assays were performed at 37°C in 5% CO_2–95% air in RPMI medium with 1% (adherence assays) or 10% (IL-8 induction) fetal calf serum. To assay bacterial adherence, AGS cells were grown to confluence on coverslips in 24-well plates, and bacteria were allowed to adhere for 2 h in a total volume of 1 ml. Cultures were washed five times and stained with Wright-Giemsa stain. For IL-8 induction, AGS cells were grown to confluence in 6-well plates (1×10^6 to 3×10^6 cells/well at the time of infection). Kato III cells were seeded at 0.4×10^5 to 1.0×10^5 cells/well into 96-well plates just before infection. Bacteria at the doses shown in Fig. 4 were added in a total volume of 1 ml for AGS or 0.2 ml for Kato III cultures. Supernatants were collected 6 to 8 h after infection, and IL-8 concentrations were determined by enzyme-linked immunosorbent assay, as described previously (12). Briefly, goat anti-human IL-8 (R & D Systems, Minneapolis, Minn.) was used as the capturing antibody and rabbit anti-human IL-8 (Endogen, Cambridge, Mass.) was used as the detecting antibody. The secondary antibody used was peroxidase-labeled goat anti-rabbit immunoglobulin G (Biosource International, Camarillo, Calif.).

To determine bacterial viability, bacteria were washed with 0.9% saline, incubated with proprietary fluorescent dyes from the Live/Dead BacLight kit (Molecular Probes, Eugene, Oreg.) for 15 min, and observed under a fluorescent microscope. In cultures containing >95% coccoid organisms, >90% of the bacteria stained green with the BacLight kit, indicating an intact membrane (viable bacteria stain green, while dead bacteria stain red since they cannot exclude a red fluorescent DNA-staining dye). In addition, viability was determined by the ability of the bacteria to reduce the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (13). In this assay, 10^7 coccoid or spiral *H. pylori* organisms were pelleted and resuspended in 50 μl of brucella broth containing 10 mM MTT and then incubated at 37°C for 2 h.

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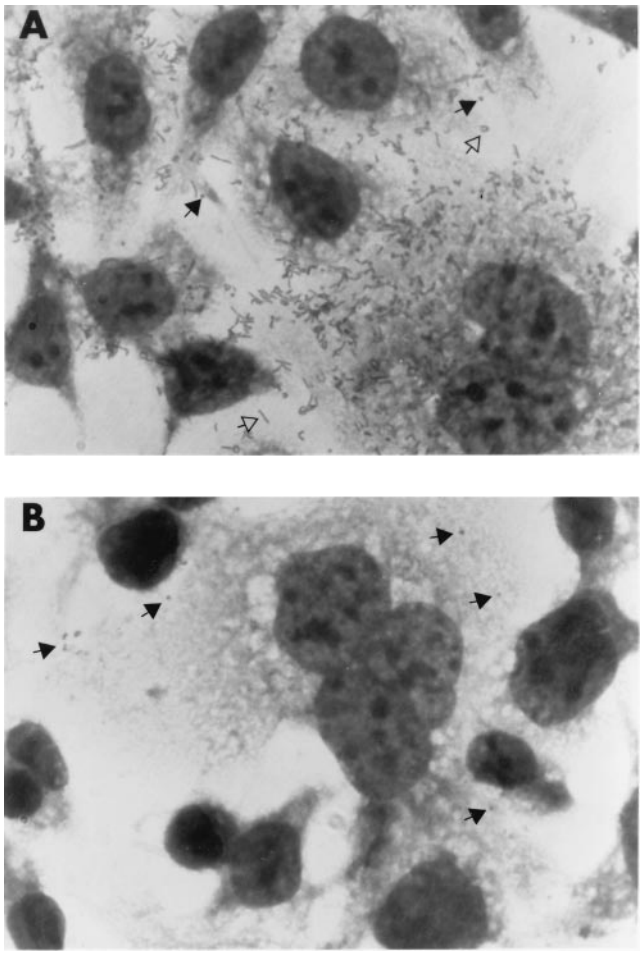

FIG. 1. Adherence of spiral and coccoid *H. pylori* to AGS human gastric epithelial cells. Confluent AGS monolayers on coverslips were infected with 5×10^7 *H. pylori* cells per ml, containing either 90% spiral (A) or ≥95% coccoid (B) organisms. Cultures were incubated for 2 h, washed, and stained with Wright-Giemsa stain. Solid arrows indicate coccoid *H. pylori*, and open arrows indicate spiral or curved *H. pylori*. Note the large numbers of spiral bacteria adhering to AGS cells in panel A compared to the few coccoid bacteria adhering in both panels.

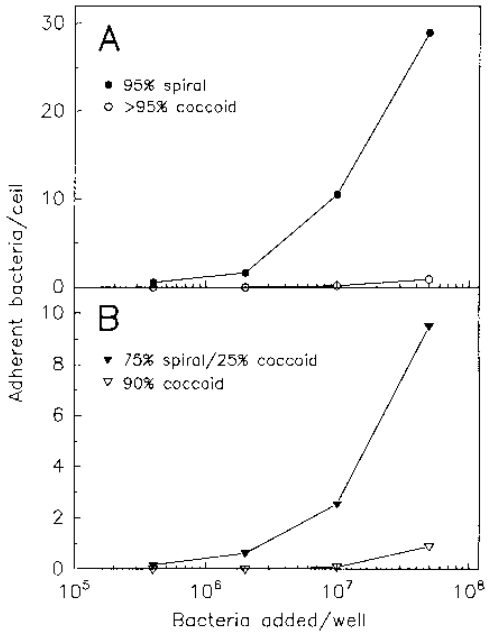

FIG. 2. Increased adherence of the spiral form of two *H. pylori* strains to AGS gastric epithelial cells. Cultures of *H. pylori* SD 4 (A) or SD 14 (B) were incubated with confluent AGS cells on coverslips, washed, and stained with Wright-Giemsa stain. The numbers of bound bacteria were then quantitated by light microscopy. Values represent means of two experiments.

Cultures treated with 4% formalin for 1 min were used as a control for dead bacteria. After pelleting, MTT-formazan was extracted by incubating bacteria overnight in isopropanol. The cell debris was pelleted, and supernatants were analyzed at 550 nm. In triplicate experiments, bacteria in cultures containing 90% coccoid and 10% spiral organisms had only 1.8-fold lower respiratory activity (reduction of MTT per bacterium) than bacteria in cultures containing 95% spiral and 5% coccoid organisms, indicating that the coccoid *H. pylori* used in this study was viable.

We first determined the ability of spiral and coccoid *H. pylori* to adhere to gastric epithelial cells. As shown in Fig. 1 and 2, coccoid *H. pylori* adhered poorly to AGS gastric epithelial cells, compared with the spiral form of the identical clinical isolates. A similar finding was made with Kato III cells (data not shown). Moreover, we found that regardless of the bacterial form, bacteria which adhered to Kato III cells had intact membranes, as determined by the BacLight kit. In contrast, dead bacteria were not observed adherent to epithelial cells. Figure 3 shows that the adherence of coccoid or spiral *H. pylori* at a constant bacterium/cell ratio did not increase significantly after 2 h.

Further experiments characterized the ability of coccoid and spiral *H. pylori* to induce IL-8 secretion by Kato III and AGS gastric epithelial cells. As shown in Fig. 4 for two clinical *H. pylori* isolates, coccoid bacteria induced little, if any, increase in IL-8 secretion by these cells. In contrast, spiral *H. pylori* increased IL-8 secretion >30-fold in Kato III cells and >150-fold in AGS cells. The increase in IL-8 secretion following addition

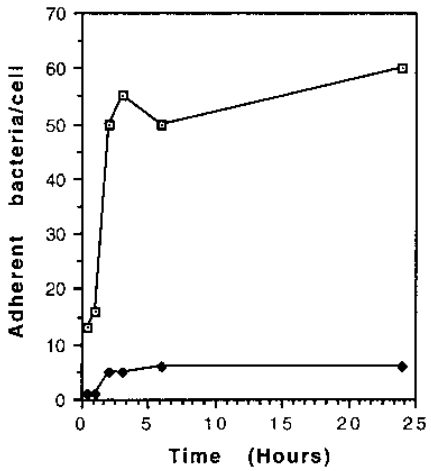

FIG. 3. Time course of adherence of coccoid and spiral *H. pylori* to AGS cells. Cultures of strain SD 14 that were >95% coccoid (diamonds) or >95% spiral (squares) were incubated with confluent AGS cells on coverslips for various time periods. Both coccoid and spiral bacteria were inoculated at 5×10^7 cells/ml. The number of adherent bacteria per cell was determined microscopically following Wright-Giemsa staining.

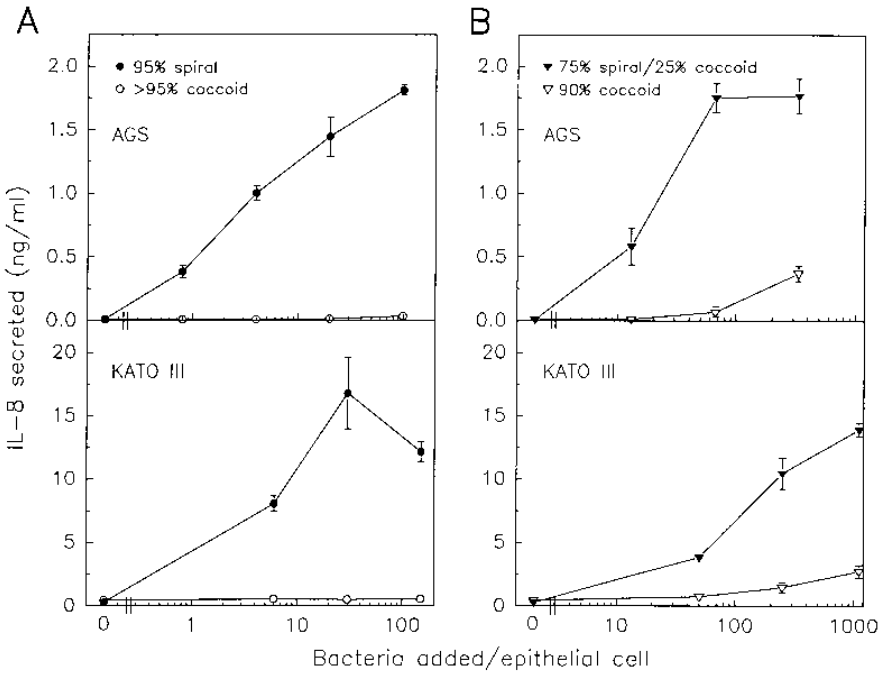

FIG. 4. Increased IL-8 secretion by human gastric epithelial cells following infection with spiral, but not coccoid, *H. pylori*. Cultures of AGS and Kato III cells were infected with the indicated doses of *H. pylori* SD4 (A) or SD 14 (B) and incubated for 6 to 8 h, and IL-8 concentrations in the supernatants were determined. Data are means ± standard deviations of the results of triplicate culture wells.

of spiral *H. pylori* was dependent on the bacterial inoculum and was maximal at a bacterium/cell ratio of 30:1 to 1,000:1.

Our findings that the coccoid form of *H. pylori* is less efficient than the spiral form in adhering to gastric epithelial cells and that it has a reduced ability to induce IL-8 secretion have important implications for the pathogenesis of the infection. Conversion of the spiral to the coccoid form, e.g., by environmental factors or antibiotic treatment (1, 3, 6), may allow the bacteria to remain in the host in a "silent" state that does not induce an inflammatory response. Alternatively, if an inflammatory host response is important for providing nutrients to the bacteria, as has been suggested (2), the coccoid forms would be less likely to persist in the host. In addition, reduced adherence of the coccoid form to epithelial cells suggests that the organism either may be shed in the coccoid form or may be less dependent on epithelial adhesion for survival.

The mechanisms underlying the reduced ability of coccoid *H. pylori* to induce IL-8 secretion are unclear. If adherence is required for this response, reduced IL-8 secretion by the less adherent coccoid form would be expected. However, since some studies found an IL-8 inducing activity in supernatants from overnight cultures of *H. pylori* (14), it is also possible that the coccoid form produces less of this kind of activity. Regardless of these mechanistic considerations, our findings that >50-fold more coccoid than spiral organisms are required for the same level of adherence and IL-8 induction are likely relevant in vivo, since coccoid *H. pylori* is, on average, not more abundant than spiral *H. pylori* in biopsy specimens from the stomach and duodenum (18).

The role of the coccoid form in *H. pylori* infection is controversial. Most investigators have not been successful in culturing coccoid *H. pylori*, but it does synthesize DNA (3, 21), and one group has reported successful regrowth of coccoid cultures (21). Furthermore, the coccoid form is considered nonmotile, which may be a disadvantage for colonizing the gastric mucosa (20). However, coccoid *H. pylori* has flagella (3), raising the possibility that it could be motile under some circumstances. Finally, studies from animal models have also yielded contradictory results, since gnotobiotic piglets could not be infected with coccoid *H. pylori* (11), while mice could be colonized by these organisms (5).

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