Immunoglobulin G Antibody Response to Infection with Coccoid Forms of *Helicobacter pylori*

G. Figueroa,* G. Faúndez, M. Troncoso, P. Navarrete, and M. S. Toledo

Laboratory of Microbiology, Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

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An increasing number of studies support a potential role for coccoid forms in *Helicobacter pylori* infection. Evidence for this was obtained through scanning microscopy, genetic analysis for virulence traits, examination of the presence and activity of key enzymes, and other methods. We studied the serum immunoglobulin G responses to coccoid *H. pylori* forms by enzyme-linked immunosorbent assay (ELISA) and immunoblotting and compared them with those of bacillary cells. Sera from a total of 295 infected individuals were studied; these included sera from 100 patients with duodenal ulcers, 98 patients with nonulcer dyspepsia, 11 patients with gastroduodenal cancer, and 86 asymptomatic individuals. Initially, we characterized and selected coccoid and bacillary antigenic preparations by one-dimensional (1-D) and 2-D gel electrophoresis and immunoblotting. Data showed that coccoid and bacillary preparations with similar protein contents have similar patterns in 1-D and 2-D electrophoresis gels and antigenic recognition at blotting. These results revealed that coccoid and spiral antigens in ELISA can equally recognize specific antibodies to *H. pylori* in sera from infected individuals. The analysis of the spiral and coccoid preparations by Western blotting showed no major differences in antigen recognition. No specific bands or profiles associated with a single gastric condition were identified.

*Helicobacter pylori* spiral-coccoid dimorphism is observed both in vivo and in vitro. It is generally accepted that the coccoid forms arise as a response to stress conditions, e.g., in vitro aerobiosis (4), temperature changes (31), extended incubation (32), and in vivo antibiotic treatment (2).

Since they were first described, coccoid *H. pylori* forms were considered an irreversible phase that leads to cell death (18). Present knowledge suggests that coccoid cells are not dead but actually dormant (6, 13). Coccoid forms may therefore play a role in the survival, and eventually in the transmission, of this microorganism.

A number of reports suggest that coccoid forms maintain cell structures, metabolism, DNA indemnity (2, 24, 26, 32, 33, 34), and gene expression (25). There are also reports indicating that *H. pylori* cells are able to survive for prolonged periods in the environment, especially in water (31) and under conditions of starvation (27). This would not be surprising if we took into account that coccoid forms are biologically important for other pathogenic bacteria, such as *Vibrio vulnificus* (29) or *Campylobacter jejuni* (15). This study of coccoid forms may help us to better understand the natural history of *H. pylori* infection.

*H. pylori* infection induces a strong local inflammatory response which often is insufficient to eradicate the pathogen, and this failure may be responsible for the chronicity that these gastric diseases often demonstrate. It is not fully understood how the immune system is involved in clinical outcomes. One point upon which investigators agree is that the presence of specific antibodies can be used as an epidemiological indicator of infection (9, 12). Some studies suggest that noninvasive serologic tests may be of value to confirm treatment success (10, 17, 30). Although many studies have focused on the impact of bacillary *H. pylori* cells on immune status (1, 8, 16, 20), there is no information on the potential role of coccoid forms. The aim of this work was to study the immunoglobulin G (IgG) immune response of colonized individuals against coccoid *H. pylori* forms and compare it with that elicited by its spiral counterpart.

**MATERIALS AND METHODS**

**Strains.** We studied 21 strains of *H. pylori* isolated in our laboratory from gastric biopsy samples of Chilean adults. The isolates were confirmed through microscopy, culture, and rapid urease testing.

**Antigen preparation.** All strains were grown under microaerophilic conditions at 37°C on chocolate agar and a Skirrow antibiotic pool. Spiral cells were collected after 3 days in phosphate-buffered saline (PBS). The coccoid cells were harvested after 30 days at room temperature under aerobic conditions. Coccoid morphology was confirmed by Gram stain (100 fields) and by the strains’ inability to grow in appropriate conditions. The coccoid and bacillary antigens were prepared by the acid glycine extraction method (22), standardized in their protein content (Bio-Rad Labs, Hercules, Calif.), and maintained frozen (−20°C) until analysis.

**SDS-PAGE antigen characterization.** *H. pylori* proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); with 4 and 7% stacking and running gels, respectively. The bands were visualized with silver stain, and the gels were analyzed by Quantity One software (Bio-Rad).

**2-D electrophoretic antigen characterization.** The *H. pylori* preparations were first separated by isoelectric focusing according to a procedure described by Celis et al. (5). Antigens (200 μg/capillary) were incubated at room temperature with 40 μl of lysis solution (9.8 M urea, 2% NP-40, 2% amphotylc 3:10, 100 mM dithiothreitol) for 15 min. Preparations were loaded into the capillaries and covered with 20 μl of overlay solution (8 M urea, 1% amphotylc 7/9, 5% NP-40, 100 mM dithiothreitol). Gels were run at 200 V for 2 h, 500 V for 2 h, and 800 V for 16 h in two-dimensional (2-D) electrophoresis equipment (Protein II; Bio-Rad). After an electrophoretic run under similar conditions, the protein spots were visualized by silver staining and analyzed by 2-D Bio-Rad software.

**Western blot antigen analysis.** The *H. pylori* coccoid and bacillary antigens were evaluated by Western blot analysis (3). In brief, strips were blocked with skimmed milk, confronted with 1:150 serum dilutions, and maintained overnight at room temperature. Membranes were then incubated with an anti-human IgG

*Corresponding author. Mailing address: Microbiology Laboratory, INTA, University of Chile, Av. Macul 5540, Santiago 11, Chile. Phone: 56 2 678 1474. Fax: 56 2 221 4030. E-mail: gfiguero@uchile.cl.
alkaline phosphatase conjugate (Sigma). Reaction was revealed with 5-bromo-4-chloroindolylphosphate, nitroblue tetrazolium (GIBCO), and MgCl₂ (Merck). The molecular masses of the proteins were calculated by interpolating the proteins in a curve constructed with reference markers (Bio-Rad).

**Serum panel.** The sera obtained previously (11) were from 295 colonized individuals: 100 duodenal ulcer (DU) patients, 98 nonulcer dyspepsia (NUD) patients, 11 gastric cancer patients, and 86 asymptomatic individuals. Colonization of asymptomatic subjects was confirmed by the presence of IgG antibodies to *H. pylori*.

**ELISA for IgG antibodies to spiral and coccoid preparations.** Enzyme-linked immunosorbent assay (ELISA) was previously standardized in our laboratory (10); the conditions were optimized by check board analysis. In brief, flat-bottom plates (Maxisorp; Nunc, Roskilde, Denmark) were covered with either bacillary or coccoid *H. pylori* surface antigen preparation (0.125 µg/well). After blocking with PBS containing 10% skim milk, serum diluted 1:600 was added, the plates were incubated for 2 h at 37°C, and their reactivity was revealed with anti-human IgG alkaline phosphatase conjugate diluted 1:1,000 (Sigma). Wells were washed, and the substrate was added (p-nitrophenyl phosphate; Sigma) and maintained for 30 min at 37°C. The reaction was stopped with 3 M NaOH. Results were expressed as optical density (OD) at 405 nm. All serum samples were evaluated in duplicate.

**Adsorption assay.** Four sera with high ELISA levels were tested to evaluate the presence of common antigens between both preparations. Serum samples were assayed before and after adsorption with bacillary and coccoid antigens separately. Briefly, a total of 25 µg of antigen was incubated with 400 µl of diluted sera (in PBS diluted 1/20) under gentle agitation for 1 h at 37°C and overnight at 4°C. Adsorbed sera were centrifuged at 6,000 × g, and the supernatant was saved and kept frozen at −20°C until ELISA was performed.

**Statistics.** Comparisons between values obtained by ELISA based on coccoid and bacillary forms were performed through the Student’s *t* test (STATISTICA software).

## RESULTS

**H. pylori antigens.** The coccoid and spiral preparations revealed numerous electrophoretic bands ranging from 10 to 29 and 11 to 26 bands, respectively (Fig. 1). The molecular weights were calculated to be between 6.5 and 169 for the bacillary form and 6.5 and 200 for the coccoid form. Based on the frequency of band occurrence (50% or higher), a common profile of the following approximate molecular weights of 18 bands was drawn: 6.5, 7.5, 9, 12, 14.5, 21, 27, 30, 35, 53.5, 57, 61.5, 68, 72, 78, 84, 94, and 104. Occasionally, the coccoid or spiral preparation showed a single band (200 or 66) not visible in the other form.

**2-D electrophoretic analysis.** 2-D electrophoretic analysis revealed that coccoid preparations produced fewer spots than their spiral counterparts (135 and 163 spots, respectively). The spiral preparations showed spots in a molecular weight range of 22 to 219, and the coccoid forms showed spots in a range of 25 to 245. The pI range of the protein spots was similar for both preparations, ranging from 4.5 to 8.5. Only a few strains (*n* = 3) produced some spots observed exclusively in the coccoid forms (molecular weights of 245, 149, 136, 71, and 42 with pIs of 6.5, 7.1, 7, 4.4, and 4.5, respectively). In another three strains, the intensity of the spots was higher in the coccoid form (Fig. 2).

**Western blot analysis.** All 21 *H. pylori* strains were challenged with homologous sera. We found variability in the number and intensity of bands recognized by the homologous sera. The number of reactive bands for the different strains ranged from 9 to 20 for the coccoid antigens and from 9 to 19 for the bacillary form.

**ELISA antigen selection.** Twelve out of 21 strains were selected due to the higher number of bands detected on the blot
and a higher protein content was revealed by 1- and 2-D electrophoresis. Strains with exclusive spots or higher intensity in the coccoid forms were also included (Fig. 2).

ELISA for IgG antibodies to spiral and coccoid preparations. The IgG responses to *H. pylori* against coccoid and bacillary preparations are shown in Table 1. As observed, all the serum panels tested were reactive with both the coccoid and bacillary *H. pylori* preparations. The OD reading values showed high variability, which was reflected in elevated standard deviation values. The average ELISA values for patients with different gastric conditions were similar whether coccoid or spiral capturer antigens were used. The only exception was the cancer group, which showed lower values against both coccoid- and spiral-based antigens by ELISA. When the values were compared within each patient group, results showed significantly higher OD readings for the coccoid capturer antigen (*P* < 0.05). The only exception was the NUD patients, who had similar values for both antigens (*P* = 0.6551) (Table 1).

Adsorption assay. The results of this assay showed that all the OD values decreased similarly (more than 90%) when sera were adsorbed with either coccoid or bacillary antigens.

Western blot analysis with the antigen preparation used in ELISA. A representative subset of serum samples tested (22 DU patients, 14 NUD patients, 11 cancer patients, and 21 asymptomatic individuals) was blotted against the coccoid and spiral capturer antigens used by ELISA. The results showed that sera from infected individuals recognized *H. pylori* epitopes with a wide individual variability. Accordingly, we could not find a common recognizable profile from patients with similar gastric conditions. However, some bands appeared with a higher frequency in most sera (molecular weights of ca. 104, 87, 66, 63, 61, 35, and 28) (Table 2). Some bands were recognized only among the antigens presented by the bacillary form (molecular weights of 49, 32, 30, and 19) in serum samples from ulcer patients. Other bands were recognized only in the coccoid antigen by serum samples from asymptomatic individuals (molecular weights of 171, 156, 135, 116, 104, and 32), and yet other bands were recognized by sera from the ulcer patients (molecular weights of 144, 135, and 98). Serum samples from many asymptomatic subjects had antibodies to a 77-molecular-weight antigen; this epitope was not recognized by serum samples from symptomatic patients (Table 2).

To get a general picture of the epitopes recognized by blotting, we tested the antigens applied during ELISA against four pools of six sera representing each patient group. The results showed that the four groups of sera recognized the same bands in coccoid and bacillary antigens, with some minor differences in band intensity. The common profile recognized by the four sera groups is shown in Fig. 4.

**DISCUSSION**

The amount of scientific information gained in the last 2 decades on *H. pylori* is impressive. Genetic and biochemical characteristics, and many aspects of virulence, have already been well described (7, 21). The mechanism of transmission of *H. pylori*, however, is less well understood. Much research confirms that the oral-oral route is how one becomes infected with *H. pylori* (23). This does not, however, fully explain how *H. pylori*-related infections are so widespread the world over and why the prevalence is significantly higher in less-developed

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**TABLE 1. IgG responses in OD units to bacillary and coccoid antigens in different groups of patients**

| Patient group | No. of patients | IgG response (OD units ± SD) in antigen preparation | P |
|---------------|----------------|--------------------------------------------------|---|
|               |                | Bacillary | Coccoid | Bacillary | Coccoid |
| Cancer        | 11             | 0.690 ± 0.657 | 1.061 ± 0.679 | *P*=0.0131 |
| DU            | 100            | 1.214 ± 0.676 | 1.464 ± 0.581 | *P*=0.0000 |
| NUD           | 98             | 1.432 ± 0.674 | 1.464 ± 0.622 | 0.6551 |
| Asymptomatic  | 86             | 1.148 ± 0.660 | 1.308 ± 0.628 | 0.0002 |
| Total         | 295            | 1.249 ± 0.658 | 1.404 ± 0.617 | *P*=0.000001 |
areas. One can postulate that the fecal-oral route, via contaminated water and/or foodstuffs, could be the missing link that explains the high prevalence of *H. pylori* infection in areas with poor sanitation (14, 19). In order to satisfy this theory, one must explain how spiral cells are able to survive the hostile environmental factors that do not support them. Because coccoid cells are normally present both in vivo and in vitro, these forms could explain the persistence of *H. pylori* under hostile environmental conditions.

To explore this issue, we attempted to determine whether the coccoid forms have any impact on the immune response to *H. pylori* in colonized individuals. We established a specific ELISA aimed at evaluating and comparing the immune response to coccoid and spiral forms against a panel of sera from symptomatic and asymptomatic infected individuals. The results revealed that all infected individuals either with or without symptoms were highly reactive to both forms. However, the coccoid antigen preparations usually gave significantly higher OD readings than their bacillary counterparts. The biologic significance of these differences is probably not relevant. It is tempting to speculate that the higher values found with coccoid antigen in ELISA may be due to the overexpression of one or more major epitopes in the coccoid morphology. However, the results obtained in the blotting assays make this explanation less probable. This is because we did not find any major differences in band recognition when sera were assayed against coccoid or bacillary antigens. The data from 68 serum samples analyzed indicated that the IgG response to the coccoid antigen was as strong as that to the spiral one (Table 2). Most of the bands recognized by blotting were similar to those described by Nilsson et al. (28). We found no specific bands or profiles that could be associated with a particular morphological state or specific gastric condition. Kimmel et al. (16) obtained similar results: when comparing antigenic patterns recognized in sera from different patients, they found no association of specific *H. pylori* antigens with antibodies in patients with specific gastroduodenal pathologies.

The results presented here indicate that the coccoid forms of *H. pylori* are able to induce a humoral immune response. They also show that their immune response is similar to that induced by the bacillary forms. The analysis of the antigenic preparations applied in this study by 1-D and 2-D electrophoresis and immunoblotting showed that the total protein content of the coccoid forms is very similar to that of the spiral ones. Furthermore, it established that the cell proteins remain intact, as demonstrated by their immunogenic potential. The results of ELISA with adsorbed sera support this idea. These assays using cross-adsorbed sera confirm the presence of common antigens in coccoid and bacillary preparations.

An important subject that remains to be elucidated is whether coccoid forms also elicit a cellular immune response or, in particular, whether there are differences in Th1 or Th2 involvement when coccoid antigens are presented. Future studies in our laboratory will focus on some of these issues.

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