Successful Cultivation of a Potentially Pathogenic Coccosid Organism with Trophism for Gastric Mucin

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We have devised a procedure that permits the cultivation of a gram-positive coccosid species from biopsy material obtained from the antrum of the stomachs of patients with gastric disorders. Antibodies directed against surface proteins obtained from the coccosid isolates were detected in all patients with gastric disorders examined in this study, including both *Helicobacter pylori*-infected and *H. pylori*-uninfected patients. Several of these isolates, including a prototype designated strain SL100, have been characterized in some detail. Strain SL100 exhibits urease and exceptionally high catalase activities and assumes a variety of spherical morphologies as detected by electron microscopy. This isolate expresses an adherin that binds to gastric mucin. The adherin activity was detected only after the isolate was exposed to an acidic pH, suggesting that in the natural process of infection, the low pH of the stomach unmasks a cell surface component with adherin activity. Strain SL100 grows best under a microaerophilic conditions (10% CO2, 5% O2, 85% N2), but it also grows quite well under aerobic conditions. Thus, this organism would be expected to proliferate outside of the human host as well as in the gastric mucosa. Oral infection of newborn piglets resulted in colonization of the gastric antrum and growth retardation. Preliminary taxonomic classification indicates similarity to the *Staphylococcus* DNA homology groups containing *S. colnii* and *S. xylosus*. One of us (C.K.) apparently became infected with this organism as indicated by gastric symptoms and the subsequent presence of strain-specific antisera not present in other workers in the laboratory.

A large proportion of the gastric disorders occurring in industrialized countries have been attributed to infections by *Helicobacter pylori* (11). However, some patients suffering from these symptoms are culture negative and antibody negative for *H. pylori*, and these disorders are often attributed to other causes, such as the habitual use of certain medications (11). In one study (9), an uncharacterized coccosid organism was proposed as the causative agent for gastric inflammation in some (8.5%) of the patients who underwent biopsy. In another study, Bode et al. (1) observed both the well-characterized spiral-shaped *H. pylori* and a coccosid form in a gastric biopsy specimen and proposed that *H. pylori* can assume a coccosid morphology. In these two studies, the coccosid forms could not be cultivated and for this reason no further characterization was possible. Several groups have observed that in old cultures or in an aerobic atmosphere, the motile, spiral-shaped *H. pylori* changes into a nonmotile coccosid form (2, 6, 15). It has been speculated that these coccosid forms appear under these unfavorable conditions as an infectious agent that can survive outside the human host. Experimental proof of a role of the coccosid forms in the transmission of infection has not been possible because the coccosid forms could not be cultivated in the laboratory. We have now isolated and cultivated a coccosid organism from biopsy material obtained from patients suffering from gastritis. Antibodies to the coccosid forms were detected in patients who were either culture positive or culture negative for *H. pylori*. Infection and colonization of porcine gastric antrum by *H. pylori* have been well established (3–5, 17). We report here that our prototype isolate, designated strain SL100, also infects the porcine gastric antrum and binds to porcine gastric mucin in vitro. We found that oral administratio...
the mucin was digested for 3 h at 37°C. The digested mucin was centrifuged for 30 min at 15,000 rpm with a Sorvall centrifuge and S34 rotor, and the supernatant was filtered over a Sephadex G-200 column equilibrated with 10 mM phosphate buffer (pH 7.2). The material eluting at the void volume was concentrated to 1 ml by dialysis against distilled water, and the solution was lyophilized.

Isoelectric focusing of bacterial surface proteins. Coccoid cells were suspended in distilled water, the cell density was adjusted to an OD_{600} of 2, and 1 ml aliquots were pelleted in two Eppendorf microcentrifuge tubes. The cell pellet in one tube was suspended in 1 ml of distilled water, and the pellet in the second tube was suspended in 1 ml of 10 mM HCl. After standing for 20 min, the cells were centrifuged and the pellets were resuspended in 20 mM phosphate buffer (pH 7.2), and the surface-exposed proteins were labeled with 32P as described previously (12). A labeled cell suspension was added to the gel, and the gels were cast and run on a slab gel electrophoresis apparatus. The gels were stained with Coomassie blue for visualization.

RESULTS

Isolation of the organism from biopsy material. We isolated a coccoid organism from the antrum region of the stomach of patients with gastric disorders by using endoscopy, and we cultured the organism on a standard blood agar medium which is commonly used to culture gastric organisms such as _H. pylori_. The culture was successful when the biopsy samples were partially embedded in blood agar plates. A ring of bacterial growth occurred around the embedded biopsy samples in 2 days. In contrast, there was no growth when the biopsy sample was ground into fine pieces and agitated vigorously before being spread on a culture plate. Similar growth was observed when the organism was spotted onto a plate at a high density (10 μl; OD_{600} = 10) but not at low density (10 μl; OD_{600} = 0.1). We suspect that the coccoid cells were present in clusters in the unbroken biopsy samples as judged by the clusters of coccoid cells seen in the antral mucosa of an infected piglet and in the culture on blood agar (Fig. 1). Taken together, these observations suggest that the organism grows best when cells are close to each other, as in cell clusters or in a heavy inoculum.

The organism exhibits strong urease and catalase activities, as does _H. pylori_. Presumably urease enables the gastric microorganism to survive in an acidic environment by producing ammonia (7, 16) while catalase enables the organism to overcome the assault of peroxides, which arise from the oxidative burst by host macrophages and neutrophils recruited to the submucosa underlying the infected mucosa (8, 14).

Scanning electron microscopy. Scanning electron microscopy revealed that the organism assumes a variety of morphologies. A spherical shape was most often observed, but in older cultures some of the spheres appeared to be dented (Fig. 1a), and in some cases the dents became so deep that the organism appeared doughnut-shaped (Fig. 1b). Occasionally, the coccoid forms were observed singly, but more frequently, they were seen in chains or clusters. When they were in a chain, it appeared as if a number of coccoid forms were encased in a stretchable outer coat, which held individual coccoid forms together.

Experimental infection of newborn piglets. A porcine system has been well established as a valid model for human infection with _H. pylori_. Therefore, we used the porcine system as a model for the human system for studying infection by our coccoid isolate. Two newborn piglets were given the coccoid-form organisms orally. At 4 days postinfection, one of the infected piglets was sacrificed, and tissue sections were made from the stomach. The tissue sections were washed and then from an individual patient; the serum was diluted 100-fold with PBS. After several washes with PBS, the strips were incubated with a commercial horseradish peroxidase-linked goat anti-human immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) which was diluted 1,000-fold with PBS. The strips were washed with several changes of PBS solution, and the protein bands recognized by serum IgG of the patients were detected by incubating the strips in a PBS solution containing 0.05% diaminobenzidine and 0.03% H₂O₂.

Serological testing and cultivation of organisms. The absence or presence of serum antibody to _H. pylori_ (IgG and IgG⁰, respectively) was measured as specified by the supplier of the diagnostic kit (Bio-Rad; no. 4404-2002). To determine the presence or absence of _H. pylori_ in the gastric biopsy material, finely macerated biopsy material was cultured on blood agar plates under microaerophilic conditions (as described above), and the translucent colonies that appeared after 5 days were examined for the presence of spiral motile organisms with strong urease activity. For positive identification of _H. pylori_, aqueous extracts of the colonies were analyzed by SDS-PAGE for the presence of the 61- and 30-kDa urease subunits as described previously (13). Fatty acid analysis with the MIDI/Hewlett-Packard microbial identification system and metabolic characterization with the Biolog identification system were carried out by Analytical Services, Inc. (Williston, Vt.).
fixed with glutaraldehyde. When the fixed tissue sections were examined with a microscope, the mucin layer of the antrum sections was found to be heavily infected with the coccoid forms, but no other organism was detected in this layer. The coccoid forms were present in patches and in large clusters, rather than uniformly dispersed, on the mucin layer of the antrum. The growth of the two infected piglets was severely retarded compared to that of uninfected littermates. At 4 days after birth, the two infected piglets weighed 0.61 and 0.64 kg, while the average weight of the six uninfected littermates was $0.96 \pm 0.07$ kg.

**Acid-dependent binding to porcine mucin.** After observing a heavy infection of the porcine antrum by the coccoid organisms, we considered the possibility that colonization depends on the low pH prevailing in the stomach. Therefore, we examined the effect of acid exposure of the coccoid forms upon their binding to porcine mucin. To detect cell-bound mucin, horseradish peroxidase was covalently linked to the mucin preparation. As shown in Fig. 2, the coccoid-form bacteria bound to porcine gastric mucin only if they were preexposed to a pH of 3.5 or lower, which approaches the prevailing pH of the human and porcine stomach (3, 18). The binding of acid-treated coccoid forms to mucin was also dependent on the ambient pH. Thus, the acid-treated coccoid forms bound avidly to the mucin preparation at pH 5, but the binding of the same coccoid forms to the mucin preparation at pH 7.0 was greatly diminished. With the present experimental protocol, it was technically difficult to carry out the binding assay at a pH below 5 because the unbound horseradish peroxidase-linked mucin preparation tended to flocculate and coprecipitate with the coccoid forms at these pH values.

**Detection of surface proteins after acid treatment.** To examine whether acid treatment of the coccoid forms disrupts their surface organization, thereby unmasking an adhesin, the surface-exposed proteins of acid-treated and untreated coccoid forms were compared. For this purpose, the cell surface proteins of acid-treated and untreated coccoid forms were labeled with $^{125}$I by the lactoperoxidase method (12). As may be seen in Fig. 3, acid treatment of the coccoid forms exposed at least four additional proteins with molecular masses of 17, 19, 25, and 34 kDa.

The gastric coccoid isolates have an exceptionally strong outer integument. Boiling strain SL100 with 0.5% SDS during extraction of $^{125}$I-labeled cell surface proteins did not alter the integrity of the cells when examined under the microscope, and no lysis was detectable as evidenced by the absorbance at 260 nm of the medium. Cells treated with 10 mM HCl for 20 min grew equally well as did untreated cells when grown on blood agar medium.

**Binding to porcine gastric mucosal scrapings.** Various coccoid organisms, including a number of species of *Staphylococcus* and *Streptococcus*, are known to colonize the pharyngeal and esophageal mucin of warm-blooded animals. However, colonization of gastric antrum by these well-characterized
coccii has not been reported. To see if any of these cocci can bind to the antrum at an acidic pH, scrapings of mucosa prepared from the antrum of a 7-day-old piglet were incubated with our gastric coccoid forms or with \textit{S. aureus} at pH 3.5. The results of this experiment (Fig. 4) seem to indicate that (i) our gastric coccoid forms bind mainly to the mucin but not to the epithelial cells of the piglet gastric antrum and (ii) \textit{S. aureus} binds neither to mucin nor to epithelial cells of the antrum at pH 3.5.

**Octylglucose extraction of surface proteins.** Since microbial species exhibit characteristic profiles of cell surface proteins, we examined the cell surface protein profiles of the coccoid isolates. Figure 5 shows protein profiles of octylglucose extracts from two isolates of the coccoid forms (lanes 3 and 4) after SDS-PAGE and Coomassie blue staining. Lanes 1 and 2 show, as controls, protein profiles of \textit{S. aureus} and \textit{Streptococcus faecalis}, respectively. The profiles of octylglucose-extractable proteins...
proteins were qualitatively similar for these two independent coccoid isolates and are distinguishable from the patterns seen for *S. aureus* and *Streptococcus faecalis*.

**Western blot analysis with serum from *H. pylori*-positive and -negative patients with gastric disorders.** To determine if the coccoid organism is associated with gastric disorders, we tested antisera from patients with gastric ailments in a Western blot analysis with octylglucose-extracted proteins of a representative gastric coccoid isolate (strain SL100). From a group of 18 patients with gastric disorders, we selected the sera of 4 patients who were antibody and culture negative for *H. pylori* and the sera of 5 patients who were antibody and culture positive for *H. pylori*. Figure 6 shows the serum IgG status of these patients with respect to the protein components of the octylglucose extract made from the coccoid isolate shown in lane 4 of Fig. 5. Figure 6 clearly shows that all of these patients had serum IgG against the 34-kDa protein and the doublet of 200-kDa proteins and that the overall profiles of the serum IgG from the *H. pylori*-infected and those from the noninfected patients were indistinguishable. Figure 6 shows that the four patients who were not infected with *H. pylori* apparently had been infected with the coccoid organism as judged by the strong serum IgG response to the coccoid antigens. These observations imply that the coccoid organism might be a causative agent of gastric ailments in some patients.

**Initial taxonomic classification based on fatty acid composition and biochemical analysis.** Our preliminary classification of strain SL100 shows similarity to *S. cohnii* based on fatty acid analysis with the MIDI/Hewlett-Packard microbial identification system and similarity to *S. xylosus* based on metabolic characterization with the Biolog identification system. These two species of *Staphylococcus* are closely related to each other based on nucleic acid hybridization analyses and have been implicated as potential human and animal pathogens (10). Further studies will be required for additional classification of strain SL100 and the other representative clinical isolates. Classification attempts involving rRNA techniques have not been carried out, since the organism cannot be routinely lysed by various techniques (sonication, French press, lysozyme, etc.) alone or in combination.

**DISCUSSION**

In industrialized Western countries, *H. pylori* is now recognized as the causative agent of most cases of gastritis and of gastric and duodenal ulcers (11). It is not clear if *H. pylori* is also the primary cause of gastroduodenal ailments in the developing nations. Strain SL100 and other isolates with similar coccoid forms were obtained from patients admitted to St. Vincent Hospital, a church-supported hospital in Suwon, Korea. This hospital provides low-cost health care to patients from a lower income group who live in neighborhoods more likely to be characterized by crowding and relatively poor sanitary conditions. It is not clear why others have not previously reported the isolation of this organism. We speculate that this organism may be more prevalent among Asians or in developing countries. The patients in this study were children during the 1950s and 1960s, when more people in Korea experienced poverty and less improved sanitary conditions. In future studies, we wish to examine the relative frequencies of isolation of coccoid forms compared to *H. pylori* and the presence of antibodies to the coccoid form and *H. pylori* in patients from various socioeconomic groups. We do not know if others have isolated this organism from biopsy material and considered it to be a contaminant. In this context, it is important to note that the urease activity of this isolate is significantly higher than that of the typical urease-positive *Staphylococcus* species. In the standard urease test using phenol red, the pH takes 5 to 30 s to change in strain SL100, while it takes 18 to 20 h in a urease-positive *S. aureus* isolate under comparable conditions.

We report here that some of the patients in this study were infected by a coccoid organism. These observations and the effects of strain SL100 following oral administration to neonatal pigs support the notion that this coccoid organism is pathogenic for humans and pigs. It is of interest that strain SL100 binds to mucin only under acidic conditions (Fig. 2). This acid-dependent binding property would ensure that the organism colonizes only the gastric mucosa and not other organs. At pH 3.5, strain SL100 binds to the porcine gastric antrum, but under the same conditions, *S. aureus* fails to bind (Fig. 4). This indicates that typical *S. aureus* strains would not normally be expected to colonize the gastric antrum and implies that strain SL100 is not a transient resident of the gastric antrum.

Strain SL100 and other such coccoid isolates assume a variety of microscopic morphologies including spheres, dented and wrinkled spheres, and other morphologic variants. While several coccoid variants of *H. pylori* have been reported, including some similar in appearance to strain SL100, these *H. pylori* coccoid forms have been reported to be noncultivatable and noninfectious (4).

During the course of these studies, one of us (C.K.) complained of severe stomach pain, and we suspected infection by the coccoid isolate. We examined his serum for the presence of antibodies against the coccoid organism, and as controls we examined the sera of five individuals without stomach pain. The serum of C.K. but not the sera of the other five individuals reacted with several microbial proteins from the coccoid iso-
late in a Western blot. The serum of C.K. did react in control experiments with proteins extracted from *Escherichia coli*, *Streptococcus faecalis*, and *Micrococcus luteus*, suggesting that individuals at this institute were infected by common human commensals. If the coccoid isolate were a common human commensal (e.g., *S. epidermidis*), all six individuals would be expected to have antibodies against this organism. In addition, we collected mouth washings from these six individuals by vigorous tooth brushing. These samples of oral flora were collected on Millipore filters and placed on blood agar plates. Profuse growth of the oral flora resulted, but none of the organisms from any of these individuals, including C.K., has the strong urease activity typical of the coccoid isolates obtained by biopsy of the patients with gastric symptoms in this study.

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REFERENCES


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