H. pylori stimulates gastrin release from canine antral cells in primary culture

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1CURE: Gastroenterology Research, Waltham, MA -coated 24-well plates at a concentration of 1 × 106 cells/well and incubated for 2 days in a humidified atmosphere of 5% CO2-95% air at 37°C.

Gastrin release. All release studies were performed using mucosal antral cells maintained in short-term culture, which resulted in an enrichment of G cells to 8–12% of the viable cell population (9). Somatostatin cells accounted for ~1.5% and mucous cells for the remainder. Before release studies, each multiwell plate was washed twice with release medium (9). Monoclonal somatostatin antibody CURE S6 (10-7 M) was included in all experiments to rule out any indirect effect on gastrin stimulation from somatostatin cells. S6 antagonizes the effects of exogenously administered and endogenously released somatostatin in the stomach (27). Somatostatin content in the G cell preparations was <5 pmoI/106 cells (9), which is blocked by S6 at a concentration of 10−7 M (27). After the plates were incubated for 2 h at 37°C in 5% CO2-95% air with either control DMEM culture medium, live HP bacteria, or various bacterial extracts, the G cell supernatants were collected, and the cells were detached from the base of unstimulated wells to determine the cell content of gastrin (16). All release studies were performed in triplicate. Three wells on each plate incubated without stimulant indicated basal release, and three wells received 10−11 M bombesin as a positive control for gastrin release. The percentage of gastrin release was then calculated by dividing the amount of gastrin in the test supernatant by the amount remaining in the adherent cells (9).

Gastrin assay. Gastrinlike immunoactivity was measured by radioimmunoassay as described previously, using antiserum 1611 in a final titer of 1:80,000 and tracer prepared by iodination of gastrin-17 by the chloramine-T method. This assay recognizes all COOH-terminal fragments of gastrin.
longer than four residues but does not detect glycine-extended gastrin variants (25).

HP strains. Three well-characterized strains were used for these studies: 1) American Type Culture Collection (ATCC) strain 43579 (14), 2) strain 60190 (17), which is also known as ATCC 49503, and 3) strain 60190:v1, a cytotoxin-deficient derivate of 60190 (7). Strains 43579 and 60190 were initially isolated from human gastric biopsies and are positive for CagA and vacuolating toxin, whereas 60190:v1 is an isogenic mutant in which the VacA gene has been disrupted by insertion of a kanamycin resistance gene (7).

Expression of CagA+ strain 43579 was assessed by immunoblotting whole cells, using anti-CagA antiserum as described previously (6). The vacuolating cytotoxic activity of strain 43579 was evaluated by incubating HP culture supernatants with HeLa cells for 18 h and monitoring cell vacuolation with the neutral red uptake assay, as described previously (4). Exponential-phase broth cultures of each strain were prepared by growth in Brucella broth (Difco, Detroit, MI) containing 5% heat-inactivated FCS and 1% IsoVitaleX (Becton-Dickinson Microbiology, Cockeysville, MD) at 37°C in a humidified 12% CO2 incubator. Previous growth curves, assessed by phase-contrast microscopy, optical density, and bacterial colony determination, have demonstrated that exponential phase growth occurs at about 36 h when cells are incubated in the culture medium described above. The optical density of the cultures before use was determined to be 620 nm, and the organisms were centrifuged and resuspended in DMEM at a concentration of 108 bacteria/ml. Fifty microliters of this suspension were used per well of G cells, which gives a final concentration of 107 bacteria/well. The cultures were actively motile as determined by phase-contrast microscopy.

For preparation of bacterial extracts, these bacteria were cultured on Brucella agar plates containing 5% sheep red blood cells and 1% IsoVitaleX at 37°C in a humidified 12% CO2 incubator. After growth for either 48 or 72 h, the plates were swabbed into 0.15 M NaCl and the optical density (620 nm) was determined and adjusted to 1.0 (5 x 108 bacteria/well). All of the following bacterial extracts were prepared from this same number of organisms, and the soluble extracts were stored at −80°C until use.

Sonic extracts were prepared by centrifuging the bacterial suspension, resuspending the bacteria in PBS, and sonicating to complete disruption (typically using 4 bursts of 140 W each for 30 s). Intact cells and large bacterial fragments were removed by high-speed centrifugation at 45,000 g for 15 min at 4°C and 0.2-µm filtration. Water extracts were prepared following the protocol of Mai et al. (20) by centrifuging the bacterial suspension, resuspending the bacteria in sterile, double-distilled water, and vortexing vigorously for 45 s, followed by centrifugation and filtration. Finally, PBS extracts were prepared as described by Craig et al. (8). After resuspension of the bacteria in PBS, the bacteria were incubated at 37°C for 4 h, before centrifugation and filtration.

A qualitative determination of urease activity was assayed by incubating 100 µl of extract with 100 µl of urea test broth (24) overnight in a 37°C water bath. The development of a pink-red color indicated urease activity. All three of the preparations—sonic, water, and PBS extracts—were positive after overnight incubation. Protein concentrations were determined using a modification of the Lowry protein assay (21).

Responsiveness of G cells after release experiments. The viability of G cells was assessed after release experiments with HP sonicates to exclude a cytotoxic effect. The viability of G cells was determined by restimulation with bombesin, as previously described (16). Briefly, after completion of the incubation with sonic extracts, the cells were washed and incubated for an additional 4 h at 37°C in 5% CO2-95% air. G cells were then stimulated with 10−10 M bombesin for 2 h. Previously unstimulated cells were also treated with bombesin and used as a positive control.

Statistical analysis. Statistical differences were assessed by Student’s t-test. Results are means ± SE. P < 0.05 was considered significant.

RESULTS

HP live cells. There was a modest but significant (P < 0.05, n = 5) stimulation of gastrin release from canine G cells when cells were exposed to actively motile, exponential-phase cultures of strain 43579 and 60190 at a concentration of 108 bacteria/well (the highest concentration tested) (Fig. 1). Strain 43579 stimulated gastrin release by 19% above basal values, whereas strain 60190 caused a 28% increase. Strain 60190:v1 also induced gastrin secretion, although the results were not statistically significant. When tested at 106 and 105 bacteria/ml, no stimulatory effect was induced by any of the strains.

HP sonic extracts. Incubation of canine G cells with whole cell sonic extracts obtained from 48-h plate-grown cultures of each strain significantly enhanced gastrin release (P < 0.01, n = 5). There was a modest but still significant increase induced by 72-h broth cultures from strain 43579 but not the other two strains (Fig. 2). Strain 43579 stimulated gastrin release by 53% (48 h) and 37% (72 h) above basal values, strain 60190 stimulated gastrin release by 32% (48 h) and 18% (72 h) above basal values, and strain 60190:v1 stimulated gastrin release by 50% (48 h) and 3% (72 h) above basal values. The protein concentration of all sonic extracts was 0.3 ± 0.1 mg/ml (48 h) and 1 ± 0.2 mg/ml (72 h).

Water-extracted surface components. Extracts from each strain prepared by vigorous vortexing in double-distilled water were able to cause significant (P < 0.01, n = 5) stimulation of gastrin release, regardless of whether the bacteria were cultured for 48 or 72 h (Fig. 3).

![Fig. 1. Effect of Helicobacter pylori (HP) live bacteria on gastrin release. Basal gastrin release is compared with gastrin release stimulated by HP live bacteria (strains 43579, 60190, and 60190:v1). Data are means ± SE from 5 preparations. * P < 0.05. Basal gastrin release was 0.7 ± 0.1%; bombesin-stimulated release was 2.6 ± 0.3% (not shown).](http://ajpgi.physiology.org/)
3). This was all the more impressive since the preparations contained less than 10 µg of protein/ml. Strain 43579 stimulated gastrin release by 41% (48 h) and 25% (72 h), strain 60190 by 41% (48 h) and 53% (72 h), and strain 60190:v1 by 33% (48 h) and 49% (72 h) above basal values. The bacteria remained viable after water extraction as determined by phase-contrast microscopy and colony-forming unit determination.

PBS extracts. There was no effect on gastrin release when PBS extracts prepared from 48- or 72-h cultures from any of the three strains were added to the monolayers (0.7 ± 0.1% basal vs. 0.7 ± 0.1 with PBS extracts, n = 5). The protein concentration of the PBS extracts was <10 µg/ml (48 h and 72 h).

Responsiveness of G cells after release experiments. As a control for confirming cell viability after treatment with sonic extracts, the responsiveness of the treated G cells to bombesin stimulation was determined. Previously unstimulated G cells showed a 1.5-fold increase of gastrin release after bombesin stimulation, similar to cells that had been previously exposed to HP sonic extracts (n = 3; Fig. 4).

**DISCUSSION**

We report here for the first time that live HP bacteria or bacterial extracts can directly stimulate the release of gastrin from canine antral G cells. These experiments demonstrate that HP produces a soluble factor(s), probably surface exposed, which can cause G cell cultures to release enhanced levels of gastrin. This observation is consistent with the hypothesis that HP can directly contribute to the damage observed in gastroduodenal disease.

It is now well established that duodenal ulcer patients chronically infected with HP have increased basal and stimulated gastrin release. Circulating gastrin may be responsible for driving basal acid secretion, which is increased in duodenal ulcer patients (19). Recently, it was demonstrated that the fall in gastrin after HP eradication was accompanied by a proportional fall in basal acid secretion (22). Reversion of hypergastrinemia and increased basal secretion may contribute to the duodenal ulcer remission after HP eradication.

The presence of HP in the gastric antrum is associated with mucosal inflammatory cells such as neutrophils, lymphocytes, monocytes/macrophages, and plasma cells (3, 20). HP secretes a potent chemotactic factor, possibly urease, for mononuclear and polymorphonuclear inflammatory cells and stimulates monocytes to release several cytokines, including TNF-α (8, 13). In a previ-
ous study we demonstrated gastrin secretion in isolated G cells by stimulated monocytes and TNF-α (16). The inflammatory response induced by HP may in turn stimulate monocytes and TNF-α secretion, which may have an additional impact on G cell function.

Water-extracted surface proteins stimulated gastrin release in our experiments. Mai et al. (20) found that this extraction procedure had essentially no contamination by lipopolysaccharide. Because these proteins are shed from the bacterial cell, this material may be similar to that released from HP in vivo (20). HP usually does not appear to invade the mucosa, and therefore the release of soluble proteins could be an important factor in stimulating gastrin release. Our observation that actively motile intact HP stimulate gastrin release is consistent with this hypothesis.

We examined bacterial extracts at two different time points to determine whether the bacterial gastrin stimulatory factor(s) was produced primarily in the midexponential (48 h) or early stationary (72 h) phase. Although the water extracts were equally potent in gastrin stimulation using either 48- or 72-h cultures, the sonic extracts prepared at 48 h were more effective than those prepared at 72 h. Although the explanation for this remains unknown, one possibility is that cytoplasmic or periplasmic proteases produced during later growth stages may be released by sonication and may alter the gastrin stimulatory activity. Additional studies are planned that will characterize the gastrin-stimulating factor(s) present in water-extracted surface proteins from these HP strains.

Two major phenotypic characteristics known to differ among HP strains are production of a vacuolating cytotoxin (5) and the presence of a cytotoxin-associated protein encoded by CagA (6). These two related phenotypes are considered to be potentially important virulence factors that may affect the clinical outcome of HP infection (7). The mechanisms whereby CagA or vacuolating toxin could be related to the pathogenesis of ulcer disease are unknown. Restimulation of G cells with bombesin after treatment with sonic extracts demonstrated that these monolayers were still viable and intact, indicating that stimulation of gastrin release occurred without cell damage by enzymes or cytotoxic activity. In addition, disruption of the VacA gene did not influence the stimulatory effects of sonicates and water extracts on gastrin release in our experiments, suggesting that the importance of this cytotoxin as a virulence factor is unrelated to its potential to stimulate gastrin release.

Our whole organism studies provided only modest increases of gastrin stimulation over baseline at the highest level of bacteria we used (10⁸/ml) for the two VacA+ strains, whereas at lower concentrations no stimulatory effect was induced by any of the strains. HP live cells of strain 60190:v1 also induced gastrin release, although the results were not statistically significant. It is probable that at higher concentrations of the VacA-deficient strain, we would have seen significant levels of gastrin secretion. A dose-response curve with higher concentrations was not performed, because increasing bacterial numbers to 10⁹/ml or more might be much greater than physiologically reasonable.

Eradication of HP in infected patients results in increased synthesis and release of somatostatin (23). It has been suggested that suppression of somatostatin might explain the increased gastrin release in HP-infected patients (23). However, the stimulatory effect on gastrin release by HP observed in this study occurred in the presence of the somatostatin antibody S6, suggesting that HP causes a direct effect on G cell-mediated gastrin release. The stimulatory effect on gastrin release by mononuclear cells and cytokines has also been observed with S6 (1, 16). However, these observations do not exclude an additional, independent effect on somatostatin release.

We have provided evidence that three HP strains, incubated with canine G cell primary cultures as either actively motile bacteria, soluble sonic cell extracts, or water-extracted surface proteins, stimulate gastrin release. HP and cytokines may both play an important role in HP-induced hypergastrinemia.

REFERENCES


